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945/BPI

**IMPROVEMENT OF PLASMID
STABILITY IN *E. coli* FOR
INDUSTRIAL PRODUCTION**

This thesis was made at the University of Bielefeld, Faculty of Technology, The Chair of Fermentation Engineering under the guidance of Apl. Prof. Techn. Karl Friehs and prof. Božidar Šantek

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POVEĆANJE STABILNOSTI PLAZMIDA U *E. coli* ZA INDUSTRIJSKU PROIZVODNJU

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Sažetak: *E. coli* je najčešći domaćin za proizvodnju rekombinantnih proteina. Većina ekspresijskih sistema baziranih na njoj ovisi o pristupnosti barem jednog plazmida koji se treba pravilno rasporediti u stanice kćeri tijekom diobe. Segregacijska stabilnost plazmida je jedan od najvećih problema tijekom industrijske proizvodnje rekombinantnih proteina tijekom koje je potrebno izbjeći uobičajenu metodu dodatka antibiotika kulturi, a jedan od alternativnih načina je upotreba auktotrofnih mutanata transformiranih plazmidom koji uz gen od interesa sadrži gen koji komplementira mutaciju soja. U ovom radu korišten je mutiran soj *E. coli* s deletiranim genom za trioza fosfat izomerazu bez kojeg bakterija ne može rasti na glicerolu. Soj je transformiran plazmidom koji komplementira mutaciju te ima rezistenciju na antibiotik. Rast mutiranog soja i segregacijska stabilnost plazmida uspoređeni su sa roditeljskim, ne mutiranim sojem, transformiranim istim plazmidom, te uzgajanim u mediju sa i bez antibiotika. Uz to, kreiran je novi auktotrofni soj, sa smanjenom mogućnošću pojave revertanata.

Ključne riječi: *E. coli*, segregacijska stabilnost, trioza fosfat izomeraza

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IMPROVEMENT OF PLASMID STABILITY IN *E. coli* FOR INDUSTRIAL PRODUCTION

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Abstract: *E. coli* is most common host for recombinant protein production. Majority of the expression systems based on it depend on attendance of at least one plasmid that needs to be segregated into dividing cells during growth. Segregational plasmid stability is one of the major issues of industrial scale production of recombinant proteins during which conventional method of adding antibiotics should be excluded. One of the alternatives is to use auxotrophic mutants transformed with plasmid that contains gene of interest and gene that complements the mutation. In this thesis mutant strain of *E. coli* with deleted gene for triosephosphate isomerase, transformed with plasmid which complements mutation and has resistance to antibiotic, is used. Its growth and segregational stability of the plasmid is compared to the transformed parent strain, cultivated in medium with and without antibiotic. In addition, new auxotrophic strain, with lower probability of creating revertants is created.

Keywords: *E. coli*, segregational stability, triosephosphate isomerase

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1. INTRODUCTION

Production of recombinant proteins in highly purified and well characterized form has become a major task for the protein chemist within the pharmaceutical and other industries. The gram-negative bacterium *E. coli* is the most commonly used organism for heterologous protein production. Some of the reasons are that this organism is very well known and established in each laboratory, it grows rapidly and at high density on inexpensive substrates, and there is great availability of an increasingly large number of cloning vectors and mutant host strains.

Recombinant protein production requires the use of a bacterial plasmid construct as vector carrying a gene to be expressed and plasmid instability is a significant concern in the academic and industrial utilization of microorganisms for protein or DNA production. The most commonly used selectable markers to avoid the domination of plasmid-free cells, are antibiotic resistance genes. However, antibiotics are expensive compounds and they can contaminate the biomass or production product. Given that these contaminations are unacceptable from industrial and medical or regulatory perspectives it is necessary to make the assessment of the residual antibiotics levels and their subsequent removal which are costly procedures. Additionally, the use of antibiotics in intensive culture conditions (high biomass and/or continuous culture) is not efficient due to dilution or antibiotic inactivation. Several alternative strategies have been developed to reduce the risk that plasmid-free cells overtake a culture and one of those is using auxotrophic markers based on complementation of mutation on plasmid and deletion in the host chromosome.

In this thesis an alternative approach to the use of antibiotic selection markers will be explored, using an auxotrophic *E. coli* strain, that has deleted gene for triphosphate isomerase (tpiA). TpiA is a central enzyme of glycolysis and its knockout will have tremendous effect on growth of the cells. Knock-out strain will not grow on glycerol whereas growth on glucose is still possible but with lower rate than usual. It will be transformed with plasmid that complements the mutation, has an antibiotic resistance marker and has the gene for sfGFP. Its growth and plasmid stability will be observed in different volumes, from shake flasks to bioreactor, in batch and fed-batch mode, and compared to parent, non-mutated strain, cultivated in pure medium and medium with addition of antibiotic. Plasmid stability will be observed as fluorescence while plasmid carries the gene for sfGFP. In addition, new tpiA knock-out strain will be created, with lower probability of homologous recombination and creating revertants.

2. THEORETICAL BACKGROUND

2.1. *Escherichia coli*

Escherichia coli is a major bacteria of the normal intestinal flora and has huge biotechnological importance being the host for a mass-production of recombinant proteins, important for industrial as well as for pharmaceutical use. It doesn't differentiate, sporulate, fix nitrogen, photosynthesize, excrete large amounts of protein or grow in exotic environments but it is still preeminent in research laboratories for more than a century (Neidhardt, 1987). *E. coli* was discovered in the human colon in 1885 by German bacteriologist Theodor Escherich who called it *Bacterium coli*. The name was later changed to *Escherichia coli* to honor its discoverer (Hacker and Blum-Oehler, 2007).

Since the early days, scientist chose organisms that were easily accessible, not highly virulent and grew well on defined media and *E. coli* has risen up as the undeniable winner. Today we know about *E. coli* more than about any other cellular form of life (Neidhardt, 1987). Its genome was first sequenced in 1997 and large number of its enzymes and regulatory proteins have been characterized at the level of reaction mechanism, transcriptional and substrate-level regulations. Even some databases have been developed, for instance, EcoCyc, which allows easy searches for the sequence, function and regulation of nearly all *E. coli* genes and proteins (Theisen and Liao, 2017).

It is a facultative anaerobic and Gram-negative bacillus that measures around $1-3 \times 0,4 - 0,7 \mu\text{m}$ in size (Figure 1). It grows very quickly and adapts well to metabolic stress. The generation time of an *E. coli* culture is usually between 15 to 30 minutes, while most other bacteria that can be cultured have generation times between 20 minutes to an hour (Moulton, 2014; Stanbury et al., 1995). It grows at a temperature range of $10 - 40^\circ\text{C}$ and a pH of 7.2. Phylogenetically, it is a member of the Enterobacteriaceae, and is closely related to such pathogens as *Salmonella*, *Klebsiella*, *Serratia*, and the infamous *Yersinia pestis*, which causes plague (Blount, 2015).

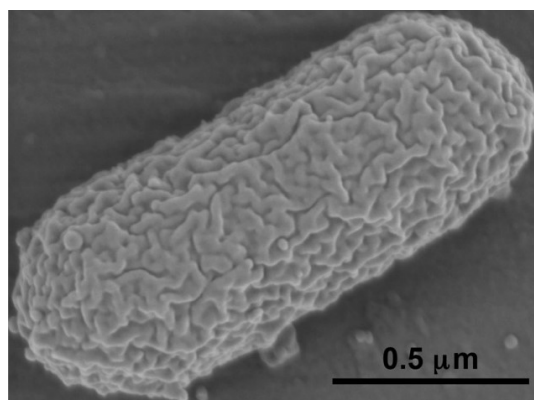


Figure 1. Scanning Electron Micrograph of *E. coli*. (Blount, 2015)

Even though it has many advantages as a microbial platform for industrial application *E. coli* has some shortcomings as well. It is sensitive to bacteriophage infection and since it is a Gram negative bacterium, its outer membrane contains lipopolysaccharide, also known as endotoxin, which evokes strong immune responses in animals. Furthermore *E. coli* production of compounds intended for human or animal consumption requires special clearance to be classified as “Generally Recognized as Safe” (GRAS). In addition, *E. coli* is able to produce many compounds but without optimization it is not the best at producing any given compound. However this situation is changing as the advanced genetic manipulation tools for *E. coli* have enabled rapid progress in optimizing *E. coli* in production of many products (Theisen and Liao, 2017).

2.1.1. Carbohydrate metabolism in *E. coli*

There are three central and constitutive routes of intermediary carbohydrate metabolism in enteric bacteria: glycolysis, pentose phosphate pathway and Entner-Doudoroff pathway (Neidhardt, 1987).

Glycolysis is the first step in the breakdown of glucose to extract energy for cellular metabolism and nearly all living organisms carry out glycolysis as part of their metabolism (Figure 2). Process is anaerobic and takes place in the cytoplasm of cells. It consists of two parts. In the first part energy is being consumed to modify and split glucose in two three-carbon molecules which are then being metabolized to pyruvate through energy releasing steps in the second part.

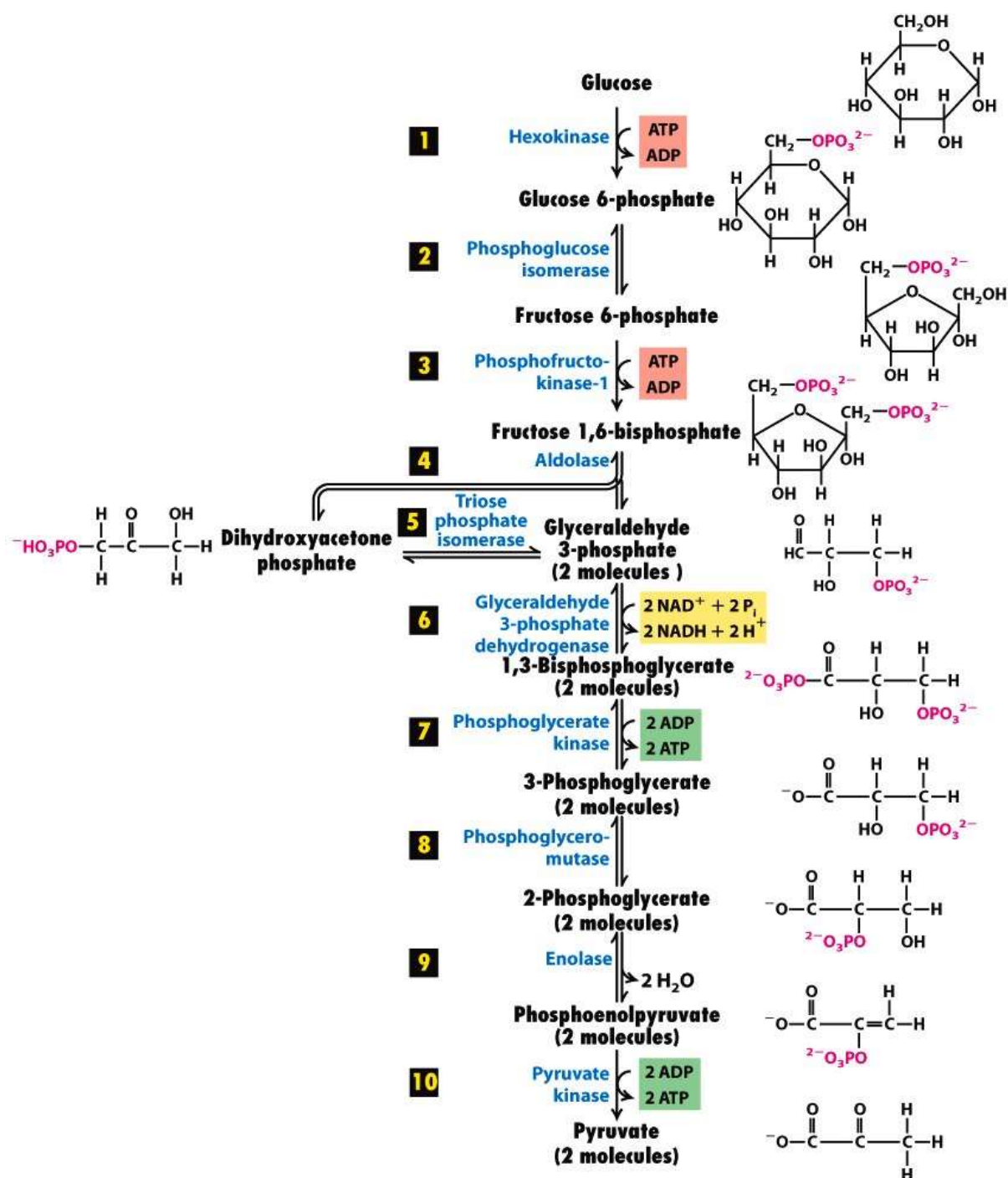


Figure 2. Glycolysis (Lodish et al., 2008)

In the last years, as a consequence of biodiesel business, glycerol has evolved as an interesting carbon source for fermentation processes. Producing biodiesel from animal fats and vegetable oils generates about 10% (w/w) glycerol as the main by-product and this may cause an issue as it cannot be disposed of in the environment. One of the possible solutions is to use glycerol as a carbon and energy source for microbial growth in industrial microbiology (da Silva et al., 2009).

Escherichia coli can utilize glycerol as the sole carbon and energy source. It is imported through the cytoplasmic membrane by a facilitator protein and it can be metabolized on two alternative pathways, depending on the growth conditions, that both end with dihydroxyacetone phosphate (DHAP). The first and also the preferred one consists of a phosphorylation step by a glycerol kinase to yield L-glycerol-3-phosphate followed by an oxidation step due to the appropriate dehydrogenase leading to DHAP. The alternative pathway consists of an oxidation step by glycerol dehydrogenase to yield dihydroxyacetone followed by phosphorylation by DHA kinase to give DHAP. In the end DHAP must be fed into the general glycolytic pathway through isomerisation by triosephosphate isomerase (tpiA) as glyceraldehyde-3-phosphate (Velur Selvamani et al., 2014).

Triosephosphate isomerase (tpiA) is a dimeric enzyme composed of identical subunits and one of the central enzymes of glycolysis (Pychersky et al., 1984). Without this enzyme cells are unable to grow on glycerol or succinate alone, but grow on succinate and other appropriately supplemented with ribose or glycerol. TpiA mutant strains are very sensitive to accumulation of dihydroxyacetone phosphate whose toxicity is related to enzymatic conversion to the bactericidal agent methylglyoxal. Although there is a glyoxylase that converts methylglyoxal to D-lactate, and thus a pathway for dihydroxyacetone phosphate dissimilation, it is of low capacity and insufficient to prevent methylglyoxal toxicity in the tpiA mutant (Neidhardt, 1987).

2.1.2. Growth kinetics of *E. coli*

Growth of an *E. coli* culture can be measured in multiple ways. Most methods rely on an estimate of cell mass by optical absorbance at a particular wavelength or on measuring the cells dry mass after centrifugation of the culture and subsequent drying of the cell pellet. The lack of these methods is the fact that you are measuring both living and dead cells. This shortage can be exceeded by plating the culture on agarose or LB plates over the lifetime but that requires a lot of work.

Typically there are four phases to the growth of a bacterial culture like *E. coli* (Figure 3). First phase is the lag phase, during which it appears that cells are not growing. It is the time of adaptation of the cells to the new environment and preparation for propagation. Cells are synthesizing needed enzymes and intermediates and consequently increasing their volume. Length of this phase depends on current level of enzymes and intermediates in the cells that are being inoculated and its relation towards level needed for the beginning of reproduction. In

commercial process the length of the lag phase should be reduced as much as possible and this may be achieved by using the right inoculum. If the cells in the inoculum are young and the new environment is favorable this phase will be really short or even completely absent.

Following comes the period during which cells grow at a constant, maximum rate and this period is known as exponential or log phase. During the exponential phase nutrients are in excess and the culture is growing at its maximum specific growth rate. This phase is being exploited in processes for production of microbial biomass and primary metabolites and it is desirable to last as long as possible.

Growth results in the consumption of nutrients, which may lead to exhaustion of some essential nutrient in the medium (substrate limitation), and the excretion of microbial products, which may generate accumulation of some toxic product. Both of the cases or their combination cause the decrease of the growth rate of the culture which results in stationary phase of growth where the growth rate has declined to zero. It is difficult to tell if the cells culture is still dividing and dying at the same rate or the culture as a whole has stopped growing and dividing. The stationary phase is misnomer in terms of the physiology of the organism as the population is still metabolically active during this phase and may produce secondary metabolites which are not produced during exponential phase. Additionally cells change their composition. Nucleic acid share is decreasing as well as the spare ingredients are accumulating.

As the growth of the culture continues late into the stationary phase, the culture will eventually reach a phase of decline, also known as death phase, where carbon sources are exhausted and/or autotoxins are inhibiting transcription and/or translation. Abrupt speed depends on microorganism and environmental conditions (Marić and Šantek, 2009; Moulton, 2014; Stanbury et al., 1995).

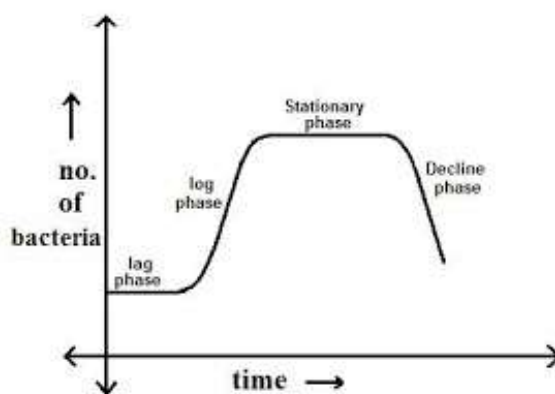


Figure 3. Growth curve of a typical microbial culture (Anonymous, 2009)

2.1.3. Recombinant protein production in *E. coli*

Proteins have multiple functions, they are catalyzers of metabolic reactions, structural components of biological assemblies, they take part in inter and intracellular interactions and cell signaling events that are critical for life. Their production plays an important role for today's medicine especially after the development of recombinant DNA technologies (rDNA) in the late 70's. In early 80's FDA approved clinical use of recombinant human insulin from recombinant *E. coli* for treatment of diabetes, being the first recombinant pharmaceutical to enter the market. Since then, other recombinant DNA drugs have been marketed in parallel with the development and improvement of several heterologous protein production systems. This has generated specific strains of many microbial species adapted to protein production, and has allowed the progressive incorporation of yeasts and eukaryotic systems for this purpose (Ferrer-Miralles et al., 2009). Bacterial expression systems for heterologous protein production are attractive because of their ability to grow rapidly and at high density on inexpensive substrates, their often well-characterized genetics and the availability of an increasingly large number of cloning vectors and mutant host strains (Terpe 2006). Among the 151 protein-based recombinant pharmaceuticals licensed up to January 2009 by the FDA and EMEA, 45 (29.8%) are obtained in *Escherichia coli*.

E. coli is the first-choice microorganism for the production of recombinant proteins, and widely used for cloning, genetic modification and small-scale production for research purposes. This is not a big surprise as the historical development of microbial physiology and molecular genetics was mainly based on this species. It resulted in a steady accumulation and worldwide use of both information and molecular tools, such as engineered phages, plasmids and gene expression cassettes. However, there are several obstacles to the production of quality proteins that limit its application as a factory for recombinant pharmaceuticals. Recombinant proteins obtained in *E. coli* lack the post-translational modifications which are present in most of eukaryotic proteins (Ferrer-Miralles et al., 2009). Another disadvantage for therapeutic use of produced recombinant proteins in *E. coli* is the accumulation of lipopolysaccharide, endotoxins, which are pyrogenic in humans and other mammals. Proteins for this application must be purified in a second step to become endotoxin-free (Petsch and Anspach, 2000).

Approved therapeutic protein-based products from *E. coli* include hormones (human insulin and insulin analogues, calcitonin, parathyroid hormone, human growth hormone, glucagons, somatropin and insulin growth factor 1), interferons (alfa-1, alfa 2a, alfa-2b and

gamma-1b), interleukins 11 and 2, light and heavy chains raised against vascular endothelial growth factor-A, tumor necrosis factor alpha, cholera B subunit protein, B-type natriuretic peptide, granulocyte colony stimulating factor and plasminogen activator. Most of the recombinant pharmaceuticals produced in *E. coli* are focused on the treatment of infectious diseases or endocrine, nutritional and metabolic disorder disease groups (Ferrer-Miralles et al., 2009).

2.1.4. Bioreactor cultivation of *E. coli*

Fermentations may be carried out as batch, continuous and fed-batch processes and the mode of operation is usually dictated by the type of product being produced.

Batch culture, as a plain example of nonstationary microbial process, is a closed culture system which contains an initial, limited amount of nutrient. Fermenter is first filled with the raw material, then sterilized and inoculated. Process is being carried on under optimum pH, temperature and aeration until the substrates are exhausted and the product is formed. Microbes in the fermenter go through lag, log, stationary and decline phases and the product remains in the fermenter until the finalization of fermentation. After the fermentation, the product is extracted and the fermenter is cleaned and sterilized before the next round. It has huge application in the industrial production, especially for the production of secondary metabolites whose production is not associated with the growth of the microbes. It is easy to set-up and the run, the risk of contamination is low, control methods are easy and quick, there is less work needed and it requires less initial investment. But on the other side you have less control over the growth of the microbes and the production of desired products, environmental conditions in the fermenter are not constant and the turnover rate is lower.

Exponential growth in batch culture may be prolonged by the addition of fresh medium to the vessel. If the medium has been designed in a way that growth is substrate limited by some component in the medium and non-toxin limited, exponential growth will proceed until the additional substrate is exhausted. Usually an overflow device is fitted to the fermenter in a way that the added medium displace an equal volume of culture from the vessel and then continuous production of cells could be achieved. If medium is fed continuously to such culture at a suitable rate, a steady state is achieved eventually, that is, formation of new biomass by the culture is balanced by the loss of the cells from the vessel. Unlike batch fermentation, in continuous fermentation process continues to run for a long period of time with the addition of nutrients and harvesting the metabolites at regular intervals. It is an open system and thus risk of

contamination is higher but it gives you more control on the growth and production and environmental conditions in the fermenter are kept constant. Turnover rate is higher and exponential growth rate of microbes is maintained. It is suitable for the production of primary metabolites like organic or amino acids. It is not easy to set-up, run and control and demands larger initial investment and labor demand.

The term fed-batch culture was introduced in 1973. to describe batch culture which are fed continuously or sequentially with medium, without removing of culture fluid. A fed-batch culture is established initially in batch mode and is then fed with fresh medium which results in an increase of volume. The use of fed-batch culture by the fermentation industry takes advantage of the fact that the concentration of the limiting substrate may be maintained at a very low level, thus avoiding the repressive effects of high substrate concentration, catabolite repression and glucose effect but also to reach high densities of biomass within short cultivation time. Whenever the specific growth rate and/or product formation are not monotonic functions of the concentration of the substrate, a fed-batch fermentation may be preferable. It allows extending the duration of the process and increases productivity, product yield and substrate utilization compared to batch fermentation.

Fundamental part of fed-batch fermentation is determining the optimal feed rate of substrate. Specific oxygen-uptake rate is directly proportional to specific growth rate and the most common way of controlling the nutrient addition is to link to a feed-back control loop using a dissolved oxygen electrode as the sensing element. If the dissolved oxygen concentration declines below the set point, meaning the cells are growing exponentially and the demand for oxygen is high, then the feed rate is reduced and when the dissolved oxygen concentration rises above the set point, meaning substrate is limiting the growth, feed rate is increased. A pH electrode may also be used as a sensing unit in fed-batch control loop for the control of oxygen demand – oxygen limitation being detected by the development of acidic conditions. Other ways include controlling the feeding by determination of the specific growth rate or by measuring limiting substrate concentration (Cutayar and Pollon, 1989; Kleist, 2002; Marić and Šantek, 2009; Modak et al., 1986; Stanbury et al., 1995).

2.2. Plasmid stability

Expression systems based on *E. coli* mostly depend on the presence of at least one plasmid which has to be segregated into dividing cell during growth. The successful subdivision of plasmids on the daughter cells is a central problem in plasmid dependent expression systems

and because of that a variety of methods has been developed in order to achieve plasmid stability.

The most commonly used method is adding antibiotics into the cultivation medium and placing genes for antibiotic resistance on the plasmid carrying the target gene. All cells without plasmid in the antibiotic containing medium will be killed or least prevented from growth. This strategy is widely used in research but in industrial biotechnology, the addition of antibiotics can be generally excluded. Antibiotics are too expensive to be used in industrial scale production and elimination of antibiotics from media and waste stream may be required during downstream processing. Plasmids without antibiotic resistance genes are especially preferred for gene therapy or gene vaccination. The main reason is that if the resistance genes are expressed in the human body it may lead to unwanted side effects. Another reason is that resistance genes may be transformed via horizontal gene transfer onto pathogenic microorganisms and making them resistant to the particular antibiotic. This is a growing problem today, especially in hospitals, and will be discussed later in this thesis. Another growing problem are allergic reactions, which could be observed in animals when plasmid DNA is used for vaccination. Furthermore, resistance is sometimes based on producing destructive enzymes to neutralize antibiotics. For instance, resistance to β -lactam antimicrobial agents, like Ampicillin, in *E. coli* is primarily mediated by β -lactamases, which hydrolyze the β -lactam ring and thus inactivate the antibiotic (Brinas et al., 2002). Gene for β -lactamases is often placed on plasmids, to make the plasmid-containing bacteria Ampicillin resistant. But that brings up a new problem. β -lactamases are mostly secreted into the medium and degrade the Ampicillin added to it so stability pressure gets lost quite fast. Therefore, other methods for plasmid stabilization are in high demand.

Another simple strategy in trying to keep a high segregational plasmid stability is using plasmids of high copy number and expecting that the statistical distribution of plasmids during cell division may always yield cells with at least some plasmids. This works fine as long as the average plasmid copy number is homogeneous, but as soon as cells with very low plasmid copy numbers show up during high growth rates, cells might have not enough time to synthesize plasmids in high copy numbers. As they are additional metabolic burden, plasmids may be lost completely, since plasmid-free cells would gain a growth advantage in comparison to plasmid-bearing cells. If plasmid free cells arise early at the beginning of cultivation, they can overgrow the whole population. At the end, there might be a lot of biomass but no plasmid and subsequently no recombinant product.

In some cases, changes in the cultivation strategies, mainly by decreasing the specific growth rate, may lead to sufficient plasmid stability. This can be achieved by reducing the cultivation temperature or changing the carbon source of the medium. However, most methods are based on adding some stabilizing elements by genetic engineering.

Nature also has some strategies to guarantee plasmid segregation. For instance, single copy plasmids often show sophisticated stabilizing mechanisms like that based on the par-system which leads to a controlled distribution of plasmids, similar to the highly organized and controlled chromosome distribution in higher organisms. The par-system consists of at least two protein-coding genes and one special site on the plasmid for controlled distribution in the dividing cell. Other systems lead to the post-segregational killing of plasmid-free cells and need the genetic information of a toxin and its corresponding antidote, with the antidote on the target plasmid. Such a combination was recently applied for the development of a *Streptomyces* based protein expression system.

There are some special systems developed for industrial production of recombinant proteins in which production strains were mutated so that they were not able to produce an essential substance or protein. One of their essential genes was knocked out on the chromosome and was placed on a plasmid. Strain with the plasmid was then cultivated in the medium which does not contain the missing substance and therefore only those cells, who carry the plasmid, could survive and divide.

One of the first methods that work in previously described way that was developed for commercial use was the valS-system. The wild-type gene for valyl-tRNA synthetase (valS) of the host carries a temperature-sensitive mutation and the gene without mutation is placed on a plasmid. For transformation the host is grown at 30°C and cultivations of transformed strain are performed at 37°C. During cultivations mutated synthetase on the chromosome loses its function and only cells carrying the valS-harboring plasmid can survive and grow. Nevertheless, valS gene is still around on the chromosome and the selection pressure favors a recombination of the mutated valS on the chromosome and the wild type valS on the plasmid. Such a recombination produces revertants with no selection pressure by valS and leads to plasmid instability. One way to reduce the probability of this recombination is the complete knockout of the chromosomal gene but in that case of valS this would not allow to obtain viable cells for transformation and, for that reason, would not be practicable.

Another strategy is the Operator-Repressor-Titration (ORT) based on negative regulation of an essential chromosomal gene by an operator sequence allowing the binding of a constitutively expressed repressor protein. In order to allow expression of the essential gene and survive, the cell has to titrate the repressor molecules against a similar operator sequence that may be present in multiple copies on the target plasmid to be maintained. An example for this is amber nonsense mutation introduced into the essential *thyA* gene in the chromosome causing thymidine auxotrophy, that was overcome by recombinant plasmids carrying a suppressor tRNA, which allowed antibiotic-free plasmid selection and also recombinant luciferase reporter expression in eukaryotic tissues and in tumor cells.

Additional strategy is to make auxotrophic mutants in a way that auxotrophy can be overcome by supplements in the medium so that competent cells can be prepared and transformed using such a supplemented medium. This may be achieved by knockout of an essential gene for example for the synthesis of an amino acid like glycine or for some of the enzymes in essential metabolic pathways like *tpiA*. In *E. coli* JW3890-2, strain from Keio knock-out collection, the *tpiA* gene is knocked out of *E. coli* leading to an auxotrophic strain which can be cultivated in full medium, like LB, but would not grow in a medium based on glycerin, like HSG. The *tpiA* gene can then be cloned on an expression vector under the control of a constitutive promoter and transformed to the strain. This would allow transformants to grow in HSG medium and keep the plasmid through generations. The problem with this strain is that *tpiA* knockout was performed in a way that the promoter and terminator of *tpiA* gene are still present on the strains genome. This may lead to homologous recombination of the *tpiA* gene back to the genome and otherwise stated make possibility of revertants showing up. To avoid this obstacle, another *E. coli* *tpiA* knockout strain, that doesn't contain any part of the *tpiA* gene, was created in later described experiments (Baba et al., 2006; Friehs, 2004; Velur Selvamani et al., 2014).

2.3. Antibiotic resistance

Antibiotics are specific metabolic products that have high physiological activity towards the specific groups of organisms such as viruses, bacteria, fungi, protozoa and malignant tumors, while inhibiting their growth or destroying them (Šušćović and Kos, 2017). The modern era of antibiotics started when Sir Alexander Fleming discovered penicillin in 1928 (Ventola, 2015). Introduction of penicillin brought us to discovery of many classes of antibiotics which put some of the most infectious diseases under control (Penesyan et al., 2015).

Antibiotics have revolutionized medicine and saved countless lives. Their discovery was a turning point in human history. Unfortunately, use of these drugs has been accompanied by the rapid appearance of resistant strains (Davies and Davies, 2010). Antibiotic resistance is a direct result of the use of antibiotics. The greater is the volume of used antibiotics, the greater are the chances that antibiotic resistant populations of bacteria will overcome the others in the contest for survival of the fittest at the bacterial level (Gelband et al., 2015).

Understanding the mechanisms of resistance has become a serious issue over the past decades and today there is a large pool of information about how bacteria can develop drug resistance. Biochemical and genetic aspects of antibiotic resistance mechanisms in bacteria are shown in Figure 4. (Džidić et al., 2008). Bacteria resist the effects of antibiotics by

- producing destructive enzymes to neutralize antibiotics,
- modifying antimicrobial targets so that drugs cannot recognize them,
- pumping antibiotics out,
- preventing antibiotics from entering by creating a biofilm or reducing permeability,
- creating bypasses that allow bacteria to function without the enzymes targeted by antibiotics and thousand other variations (Penesyan et al., 2015).

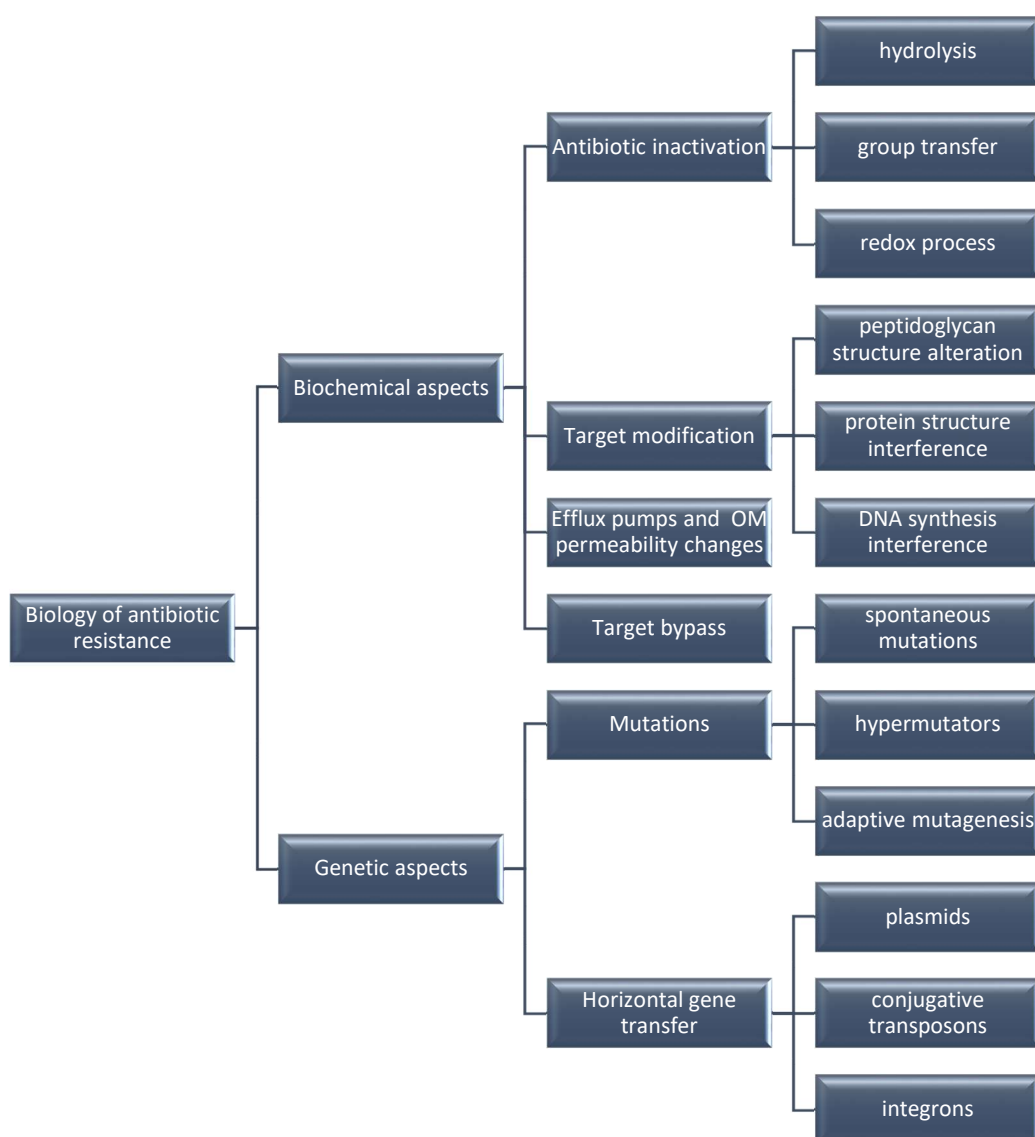


Figure. 4. Biochemical and genetic aspects of antibiotic resistance mechanisms in bacteria (Džidić et al., 2008)

Mechanisms to overcome bacterial resistance stretch from basic hygienic practices to stop the spread of bacteria in hospital to the synthesis of agents with improved antimicrobial activity (Neu 1992). Antibiotic use can be rationalized by reducing the need for antibiotics through better public health, by suppressing unnecessary use, and by improving access where use is needed (Gelband et al., 2015).

Antibiotic control programs are proven effective ways to reduce inappropriate use of antibiotics in hospitals. The responsibility of reducing resistance lies with doctor who prescribes antibiotics and with patients who demand them when the illness is viral and when antibiotics are not indicated. It is also important that the pharmaceutical industry promotes only appropriate

use of antibiotics for humans and for animals. In addition, avoiding the use of antibiotics in industrial scale production of proteins should be priority (Neu, 1992).

2.4. Green fluorescent protein

The diverse biochemical and photo physical properties of fluorescent proteins have enabled the generation of a growing palette of colors, providing unique opportunities for their use in a variety of modern biology applications (Stepanenko et al., 2011). Since its discovery in 1962 and subsequent cloning of the Green fluorescent protein (GFP) in 1994 the jellyfish *Aequorea* fluorescent proteins are the most widely used reporter proteins in all areas of biology. Due to their unique independence from cellular chaperones and non proteinogenic compounds, other than molecular oxygen, for chromophore maturation they outcompete other genetically encoded fluorescent proteins and other dyes for many *in vivo* applications (Dammeyer and Tinnefeld, 2012). The crucial breakthroughs came with the cloning of the gene and the demonstrations that expression of the gene in other organisms creates fluorescence. The gene contains all the information necessary for the posttranslational synthesis of the chromophore, and no jellyfish-specific enzymes are needed. That's why GFP has become well established as a marker of gene expression and protein targeting in intact cells and organisms (Tsien, 1998).

The structure of correctly folded GFP consists of an internal fluorophore surrounded by a tight beta-barrel (Dammeyer and Tinnefeld, 2012). Wild-type GFP is temperature sensitive and misfolds when expressed in *E. coli*. For this reason new, robustly folded version of GFP, called superfolder GFP (Figure 5), that folds well even when fused to poorly folded polypeptides, was generated. Compared to 'folding reporter' GFP, a folding-enhanced GFP containing the 'cycle-3' mutations and the 'enhanced GFP' mutations F64L and S65T, superfolder GFP shows improved tolerance of circular permutation, greater resistance to chemical denaturants and improved folding kinetics. The fluorescence of *Escherichia coli* cells expressing proteins as fusions with superfolder GFP is proportional to total protein expression (Pedalacq et al., 2006).

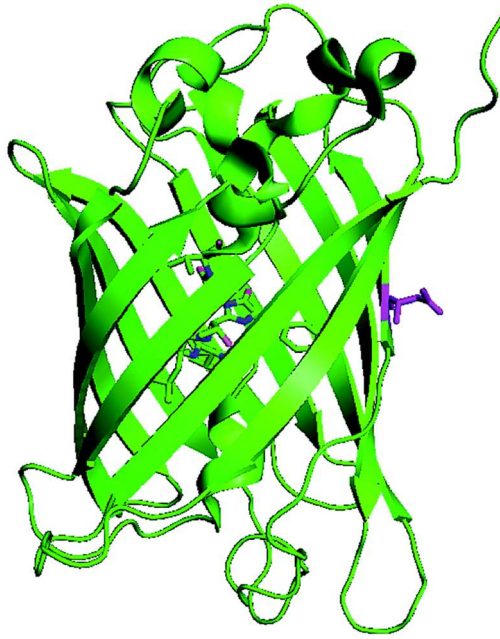


Figure 5. Structure of sfGFP (Tookmanian et al., 2015)

sfGFP absorbs light with an excitation maximum of 485 nm and fluoresces with an emission maximum of 510 nm.

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Chemicals

- 1 kb DNA Ladder, Plasmid Factory GmbH & Co. KG, Bielefeld, Germany
- Agar-agar, Carl Roth GmbH + Co. KG, Karlsruhe, Germany
- Agarose NEEO Ultra-Qualität, Carl Roth GmbH + Co. KG, Karlsruhe, Germany
- Ampicillin Natriumsalz, Carl Roth GmbH + Co. KG, Karlsruhe, Germany
- Calcium chloride, Applichem GmbH, Darmstadt, Germany
- Di-potassium hydrogen phosphate, VWR Corporation, Radnor, PA, USA
- GeneRuler DNA Ladder Mix, Thermo Scientific, Waltham, MA, USA
- Glycerin (99 %), Emery Oleochemicals GmbH, Düsseldorf, Germany
- Kanamycin sulphate, Carl Roth GmbH + Co. KG, Karlsruhe, Germany
- L-arabinose, Carl Roth GmbH + Co. KG, Karlsruhe, Germany
- Magnesium sulphate hydrate, Carl Roth GmbH + Co. KG, Karlsruhe, Germany
- Natrium carbonate, Carl Roth GmbH + Co. KG, Karlsruhe, Germany
- Natrium chloride, Fisher Scientific GmbH, Schwerte, Germany
- Natrium hydroxide, Carl Roth GmbH + Co. KG, Karlsruhe, Germany
- Phosphoric acid, Carl Roth GmbH + Co. KG, Karlsruhe, Germany
- Pluronic (antifoam), BASF SE, Ludwigshafen, Germany
- Potassium di-hydrogen phosphate, Applichem GmbH, Darmstadt, Germany
- Roti[®]-GelStain, Carl Roth GmbH + Co. KG, Karlsruhe, Germany
- Soya peptone, UD Chemie GmbH, Wörrstadt, Germany
- Tris, Fisher Scientific GmbH, Schwerte, Germany
- Tris-HCl, Carl Roth GmbH + Co. KG, Karlsruhe, Germany
- Yeast extract, Ohly GmbH, Hamburg, Germany

3.1.2. Buffers and solutions

- Ampicillin solution (100 mg/mL)

Ampicillin	100 mg
Distilled water	up to 1 mL

- Kanamycin solution (50 mg/mL)

Kanamycin	50 mg
Distilled water	up to 1 mL
- Buffer 1 (pH = 7,4)

CaCl ₂	0,1 M
Tris-HCl	2 mM
Distilled water	up to 100 mL
- Buffer 2 (pH = 7,4)

CaCl ₂	0,1 M
Tris-HCl	2 mM
Glycerin	10 %
Distilled water	up to 100 mL
- TAE BUFFER (pH = 8)

Tris	40 mM
Acetic acid	20 mM
EDTA	1 mM
Distilled water	up to 1 L

3.1.3. Culture medium

- LB (Lysogeny broth) media (pH = 7,4)

Soya peptone	10 g
Yeast extract	5 g
NaCl	10 g
Distilled water	up to 1 L

For making plates 15 g of agar-agar was added before autoclaving
- HSG media (pH = 7,4)

Soya peptone	13,5 g
Yeast extract	7 g
Glycerin (99 %)	14,9 g
NaCl	2,5 g
K ₂ HPO ₄	2,3 g
KH ₂ PO ₄	1,5 g

MgSO ₄ x H ₂ O	0,14 g
Distilled water	up to 1 L

- Feeding medium

Glycerin (99%)	527 g
Yeast extract	90 g
MgSO ₄ x H ₂ O	2 g
Distilled water	up to 1 L

3.1.4. Enzymes

- Phusion R High-Fidelity DNA Polymerase, New England Biolabs, Ipswich, MA, USA
- Dream Taq, Thermo Scientific, Waltham, MA, USA

3.1.5. Primers

Primers used for PCR during experiments were custom made by INVITROGEN, Carlsbad, Kalifornija, SAD

3.1.6. Bacterial strains

In experiments described in this thesis strain *Escherichia coli* BW25113 and its derivatives were used. It is a common laboratory strain that was created in the laboratory of B. L. Wanner as a derivative of the F⁻, λ⁻, *E. coli* K-12 strain BD792. Today it is known as the parent strain for the Keio collection, a major resource consisting of approximately 4,000 single-gene deletion mutants. Keio collection was utilized in a method taking advantage of the bacteriophage lambda red recombination system to perform gene disruptions with double-stranded PCR products. The strain and its derivatives are being used in countless laboratories for a variety of studies, including systematic phenotypic surveys and synthetic biology efforts.

Apart from the Keio parent strain, its derivative with a *tpiA* knock-out, *Escherichia coli* JW3890-2, was also used, to show how plasmid stability can be enhanced without using antibiotics. Later in text it is referred as Keio *tpiA* knock-out. In addition, new Keio *tpiA* knockout strain, was created to avoid possibility of homologous recombination which can happen between *E. coli* JW3890-2 and plasmid carrying *tpiA* gene, as described later on. Later in text it is referred as Keio new *tpiA* knock-out.

Table 1. List of used bacterial strains with their genotype and source

STRAIN	GENOTYPE	SOURCE
<i>Escherichia coli</i> BW25113	$\Delta(araD-araB)567$, $\Delta lacZ4787(::rrnB-3)$, λda^- , <i>rph-1</i> , $\Delta(rhaD-rhaB)568$, <i>hsdR514</i>	CGSC, Yale University
<i>Escherichia coli</i> JW3890-2	$\Delta(araD-araB)567$, $\Delta lacZ4787(::rrnB-3)$, λda^- , <i>rph-1</i> , $\Delta(rhaD-rhaB)568$, $\Delta tpiA778:kan$ <i>hsdR514</i>	CGSC, Yale University
<i>Escherichia coli</i> BW25113 new <i>tpiA</i> knock out	$\Delta(araD-araB)567$, $\Delta lacZ4787(::rrnB-3)$, λda^- , <i>rph-1</i> , $\Delta(rhaD-rhaB)568$, $\Delta tpiAnew:kan$ <i>hsdR514</i>	Made in Laboratory of Fermentation Technologies

3.1.7. Plasmid

All of the strains used in these experiment were transformed with pBR322-P100sfGFP-*tpiAO* constructed in the Laboratory for Fermentation technologies (Figure 6). Genes located on this plasmid are listed with description in Table 2. Plasmid contains strong constitutive promoter P100 so the expression of sfGFP does not have to be induced.

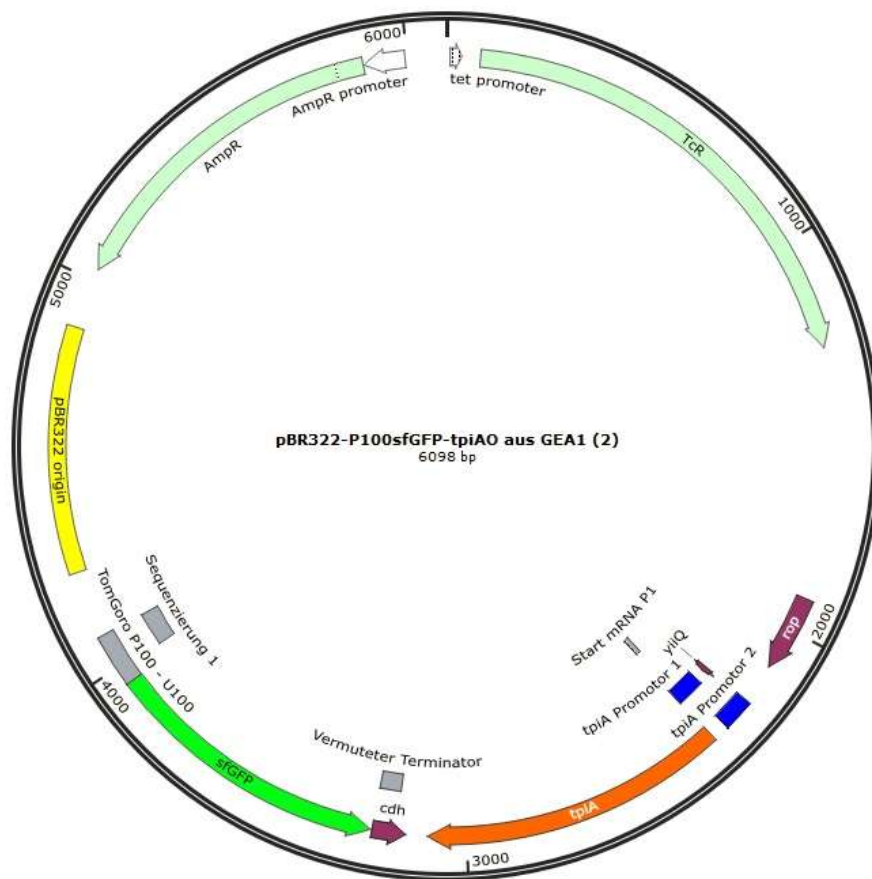


Figure 6. Map of pBR322-P100sfGFP-tpiAO made with SnapGene software, GSL Biotech LLC

Table 2. Explanation of abbreviations on plasmid map showed on Figure 6.

ABBREVIATION	DESCRIPTION
<i>pBR322</i>	origin of replication, derived from the plasmid ColE1
<i>sfGFP</i>	super folder green fluorescent protein
<i>tpiA</i>	Tri phosphate isomerase
<i>Rop</i>	Repressor of primer, keeps the plasmid number at 15 to 20 copies per cell
<i>Tc^R</i>	Tetracycline resistance
<i>Amp^R</i>	Ampicillin resistance

3.1.8. Equipment

- Agarose gel electrophoresis system MINI GEL II, VWR International GmbH, Darmstadt, Germany
- Analytical balance PM34-K DeltaRange, Mettler – Toledo GmbH, Gießen, Germany
- Autoclave D-65, Systec GmbH, Linden, Germany
- Autosampler Fraction collector FC 2038, Gilson International B.V. Deutschland, Limburg-Offheim, Germany
- Centrifuges 3-30 KS and 1-15, Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany
- Electrophoresis power supplier Standard Power Pack P25, Biometra GmbH, Göttingen, Germany
- Freezer MDF – U5386S, SANYO Electric CO., Ltd, Osaka, Japan
- Electroporator GENE PULSER II, Bio-Rad Laboratories, Hercules, Kalifornija, SAD
- Laboratory equipment (pipets, pipette extensions, petri dish, flasks, measurement jugs, beakers)
- Magnet mixer, IKAMAG REO, IKA, Staufen, Germany
- Nanodrop Spectrophotometer ND-1000, Peqlab Biotechnologie GmbH, Erlangen, Germany
- pH- meter 691, Metrohm AG, Herisau, Switzerland
- Precision balance AE 260 DeltaRange, Mettler – Toledo GmbH, Gießen, Germany
- Shaker Lab-Shaker LS – X, Kuhner AG, Birsfelden, Switzerland
- Spectrofluorometer RF-530PC, Shimadzu GmbH, Duisburg, Germany
- Spectrophotometer BioPhotometer, Eppendorf AG, Hamburg, Germany
- Transiluminator BioDocAnalyze, Biometra GmbH, Göttingen, Germany
- Ultrasound homogenizer, Sonifier 450, BRANSON Ultrasonics, Danbury, USA
- Vortex – Genie 2, Scientific Industries, Inc., Bohemia, New York, SAD

3.2. Methods

3.2.1 Cultivation

Cells were cultivated at 37 °C and pH 7,2 in different volumes, from shake flasks to fermenter. For each strain and each volume cultivation was performed two times.

3.2.1.1. Preculture setting

To grow preculture, 100 µL of glycerine culture was added to 30 mL of HSG medium and grown in 300 mL flasks without baffles. If needed 100 µL of ampicillin solution was added to the medium. Preculture was cultivated on shaker (120 rpm, 50 mm rotation radius) overnight on 37° C.

3.2.1.2. Shake flasks cultivation

100 mL of medium was added to 500 mL flask with four baffles. If needed, 100 µL of ampicillin solution, was added to the medium. Medium was inoculated with overnight cell culture to reach the start $OD_{600} = 0,2$ and flask was placed on a shaker (120 rpm, 50 mm rotation radius) at 37 °C (Figure 2). Samples for measuring OD_{600} and fluorescence and were manually taken every hour.



Figure 7. Shake flask cultivation

3.2.1.3. Batch cultivation

Bioreactor NLF 3 (Figure 8) was filled up with 5 L of medium and sterilized. Process parameters during fermentation (pH, temperature, overpressure, air flow, pO₂, stirrer speed) were automatically controlled and measured during the fermentation using software BIOSCADA Lab by BioENGINEERING. Conditions of cultivation are showed in Table 3.

Table 3. Parameter of cultivation in bioreactors

PARAMETER	VALUE
pH	7,2
Temperature	37 °C
Overpressure	0,2 bar
Air flow	5 NL/min
pO ₂	60 %
Stirrer speed	200 – 1500 min ⁻¹

pH was regulated with 10 % phosphoric acid and 2 M NaOH, which were sterilized prior to use as well as antifoam Pluronic. For control of oxygen concentration stirrer cascade was used.



Figure 8. Bioreactor connected to control cabinet and acid/base/antifoam pumps

Bioreactor was inoculated with 50 mL of overnight cell culture. Samples were taken with auto sampler every hour or every 2 hours (depending on the growth rate) aseptically using a steam-serializable sampling valve of bioreactor.

Bioreactor specification and system scheme is attached in Appendix.

3.2.1.4. Fed-batch cultivation

The same bioreactor (NLF3) and software (BiOSCADA) were used as for batch cultivation. After sterilization automatically controlled pump for feeding medium was connected to the bioreactor. Cultivation was started in the same way as in batch fermentation and feeding was controlled by pO_2 . Feeding media was being added when pO_2 fell under 60 % and it was turned off if pO_2 went under 30 %.

3.2.2. Sample analysis

Growth of the cells was measured by OD_{600} and plasmid stability by fluorescence measurement. For optical density measurement samples were simply poured into PS cuvette

and measured under 600 nm compared to blank (sterile medium). OD₆₀₀ was converted to cell concentration with formula:

$$X [g/L] = OD_{600} \times 0,39 \quad (1)$$

Specific growth rate was calculated for exponential phase of growth by using formula:

$$\mu = \frac{1}{t} \ln \frac{X}{X_0} \quad (2)$$

For fluorescence measurement cells needed to be disintegrated. Cell suspension from cultivation was disintegrated by ultrasonic lysis. 1 mL of sample (taken at each time point) was put in an ice-water bath and sonicated in three cycles for 30 seconds with 30 seconds break in between the cycles. The efficiency of sonication was monitored by microscopy.

After lysis samples were centrifuged for 5 minutes under 14 000 rpm. In the end 1 mL of supernatant was poured into PS cuvette and the fluorescence was measured through an excitation filter of 485 nm and emission filter of 510 nm.

Samples were also plated on LB plates with and without antibiotic to check their microbiological purity and appearance.

3.2.3. Transformation of bacterial strain

Keio parent and Keio knockout strains were transformed with pBR322-P100sfGFP-tpiAO previously described in chapter 3.1.7.

3.2.3.1. Making of competent cells

Competent cells are ready to use bacterial cells that possess altered cell walls by which foreign DNA can be passed through easily. There are some naturally competent bacteria but *E. coli* is not one of them. In this experiment cells were made competent with calcium chloride.

60 mL of LB-media was poured into 300 mL Erlenmeyer flask and inoculated with overnight cell culture to reach OD₆₀₀ = 0.2. It was then cultivated on the shaker at 37 °C until it reached OD₆₀₀ = 0.8 – 0.9. The culture was then transferred to plastic tubes and left on ice for 15 minutes. After cooling down culture was centrifuged for 10 minutes on 4 000 g at 4 °C. The supernatant was thrown away and cells are resuspended in 2 mL of Buffer 1. Resuspended cells were then centrifuged again on 4 000 g at 4 °C. The supernatant was thrown away again and cells were resuspended in 2 mL of Buffer 2. Cells were then portioned in Eppendorf tubes. Liquid nitrogen was used to shock freeze the cells and they were stored under -80 °C

3.2.3.2. Transformation of competent cells

In this experiment cells were transformed using heat shock treatment. For transformation 100 μ L of competent cells was melted on ice. 2 μ L of DNA was added to cells and it was incubated on ice for 20 minutes. After cooling down it was first incubated on 30 seconds on 42 °C and then it was put on ice for 2 minutes. After heat shock 500 μ L of LB media was added to suspension and it was incubated for 1 hour at 37 °C on a shaker. In the end cells were centrifuged for 2 minutes under 7 000 rpm and room temperature. 500 μ L of supernatant was thrown away and cells were resuspended in 100 μ L and plated on LB-Amp plates.

3.2.4. Disruption of a chromosomal DNA fragment

Electro component cells of the Keio parent strain were transformed with pKD46 plasmid and plated on LB – Amp plates which were put overnight at 28 °C. pKD46 carries the λ red genes behind the araBAD promoter and is temperature sensitive.

One colony was then picked from the plate and obtained in shake flask with 10 mL of LB medium with Ampicillin at 28 °C overnight. Next day 2 lid-punctured microfuge tubes with 1.4 ml of fresh LB medium with Ampicillin were inoculated with 30 μ L of the overnight culture. Tubes were then incubated on the shaker at 30 °C for 3 hours. Afterwards 50 μ L 10% L-arabinose was added to each one of the tubes to induce expression of the recombination proteins. Tubes were incubated on a shaker at 37 °C for one hour. After incubation culture was centrifuged for 30 seconds at 11 000 rpm at 2 °C and washed with chilled water 2 times. In the end cells were resuspended in 30 μ L of water.

2 μ L of prepared linear pKD13 fragment with homology arms was added to each of the two micro centrifuge tubes. The mixture was then pipetted into the chilled electroporation cuvettes. It was electroporated at 1350 V, 10 μ F and 600 Ohms. After electroporation 1 mL of LB medium without antibiotics was added to the cuvette. Cells were resuspended carefully by pipetting up and down and incubated with shaking for 3 hour at 37 °C.

After incubation culture was centrifuged for 2 minutes at 7 000 rpm. Supernatant was thrown away and the rest (around 100 μ L) was plated on LB – Kan plates (15 μ g/mL) at 37 °C overnight.

3.2.5. PCR amplification

Checkout of the disruption was performed using PCR amplification and gel electrophoresis of amplified fragment. Colonies grown on plates were picked up and suspended in 50 μL of sterile distilled water which is then used as a PCR template. Suspension for PCR was prepared as showed in Table 4.

Table 4. Suspension for PCR

Component	Volume [μL]
H ₂ O	83,5
dNTP	2
Primer 1	2
Primer 2	2
Buffer (10x)	10
Dream Taq polymerase	1

Total volume of 100 μL of suspension was proportioned in 5 PCR Eppendorf tubes. In each tube 1 μL of template was added. PCR was programmed as shown in Table 5.

Table 5. PCR procedure

Temperature [°C]	Time [min]	
95	5	
95	0,5	Loop 35x
52	0,5	
72	1	
72	5	

After amplification fragment was loaded on 1% agarose gel. Gel electrophoresis was performed for 45 minutes on 100 V. In the end gel was checked and photographed under UV light.

4. RESULTS AND DISCUSSION

The aim of this thesis was to see how certain deletions in the chromosome of the selected strains influence cell growth and plasmid stability in different cultivation volumes, from shake flasks to bioreactors. Segregational stability of plasmids is a major concern for recombinant bacterial production strains and one of the best strategies to counteract plasmid loss is the use of auxotrophic mutants which are complemented with the lacking gene along with the product-relevant ones on the plasmid. In this thesis, two strains from Keio collection were used: Keio parent strain and Keio *tpiA* knock-out. Both were transformed with previously plasmid pBR322-P100sfGFP-*tpiA*AO, later in text called pGFP-*tpiA* to make it shorter and easier to follow. pGFP-*tpiA* contains genes for *tpiA*, sfGFP, under strong constitutive promoter P100, and for Ampicillin resistance. Keio parent strain, transformed with pGFP-*tpiA*, was cultivated in HSG medium with Ampicillin and without it, while Keio *tpiA* knock-out strain, transformed with pGFP-*tpiA*, was cultivated in pure HSG medium. Both strains were cultivated in shake flasks and as a batch culture in bioreactor. In addition Keio *tpiA* knock-out, transformed with pGFP-*tpiA*, was cultivated in fed-batch culture to observe what happens in high cell density cultures over longer period of cultivation. During cultivations samples were taken every hour or every two hours, depending on growth rate, and analyzed. Every cultivation was repeated twice and the results were statistically processed. In the next chapters results are analyzed and shown graphically as a comparison of Keio parent strain, transformed with pGFP-*tpiA*, cultivated in pure HSG medium, Keio parent strain, transformed with pGFP-*tpiA*, cultivated in HSG medium with Ampicillin and Keio *tpiA* knock-out, transformed with pGFP-*tpiA*, cultivated in HSG medium. Finally new Keio *tpiA* knock-out strain was created and the results are shown in last chapter.

4.1. Fermentation in shake flasks

4.1.1. Cell growth

Growth of cells was monitored by measuring OD₆₀₀ of samples. Figure 9. shows growth curve of the Keio parent and Keio tpiA knock-out strains, transformed with pGFP-tpiA, through fermentation in shake flasks.

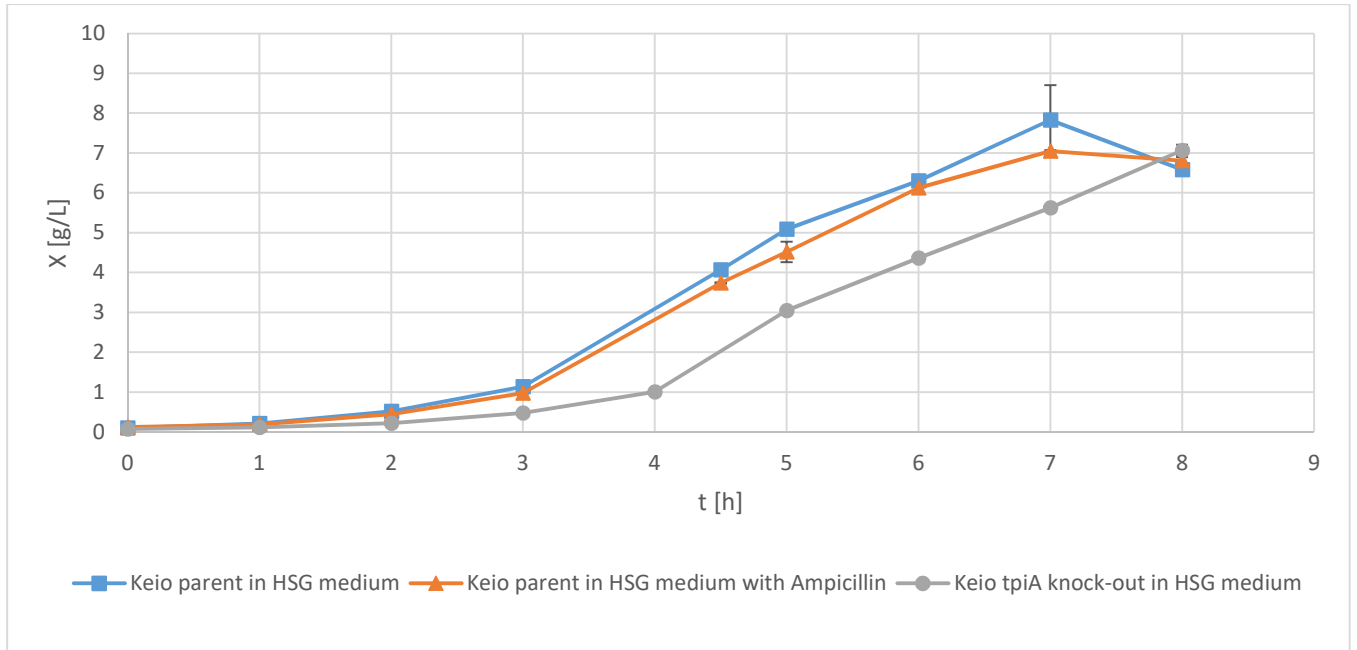


Figure 9. Growth curve of Keio parent and Keio knock-out strain, transformed with pGFP-tpiA, cultivated in shake flasks

As it can be seen from the graph, Keio parent strain, transformed with pGFP-tpiA, has similar growth in medium with and without antibiotic, with a slightly advantage while growing in medium without antibiotic. Still, all of the phases are in harmony and take the same time. If we compare the growth of the knock-out strain, transformed with pGFP-tpiA, to the transformed parent strain, its lag phase is one hour longer, and it is still in the exponential phase at 8. hour of growth. This is logical as a knock-out strain is ‘damaged’, and needs more time for growing. Cell density reached after 8 hours of cultivation is similar: 7 g/L for Keio knock-out strain, transformed with pGFP-tpiA, and Keio parent strain, transformed with pGFP-tpiA, in medium with Ampicillin, a little bit higher for Keio parent strain, transformed with pGFP-tpiA, in medium without antibiotic.

Comparison of specific growth rate between the strains during cultivations in shake flasks are shown on Figure 10.

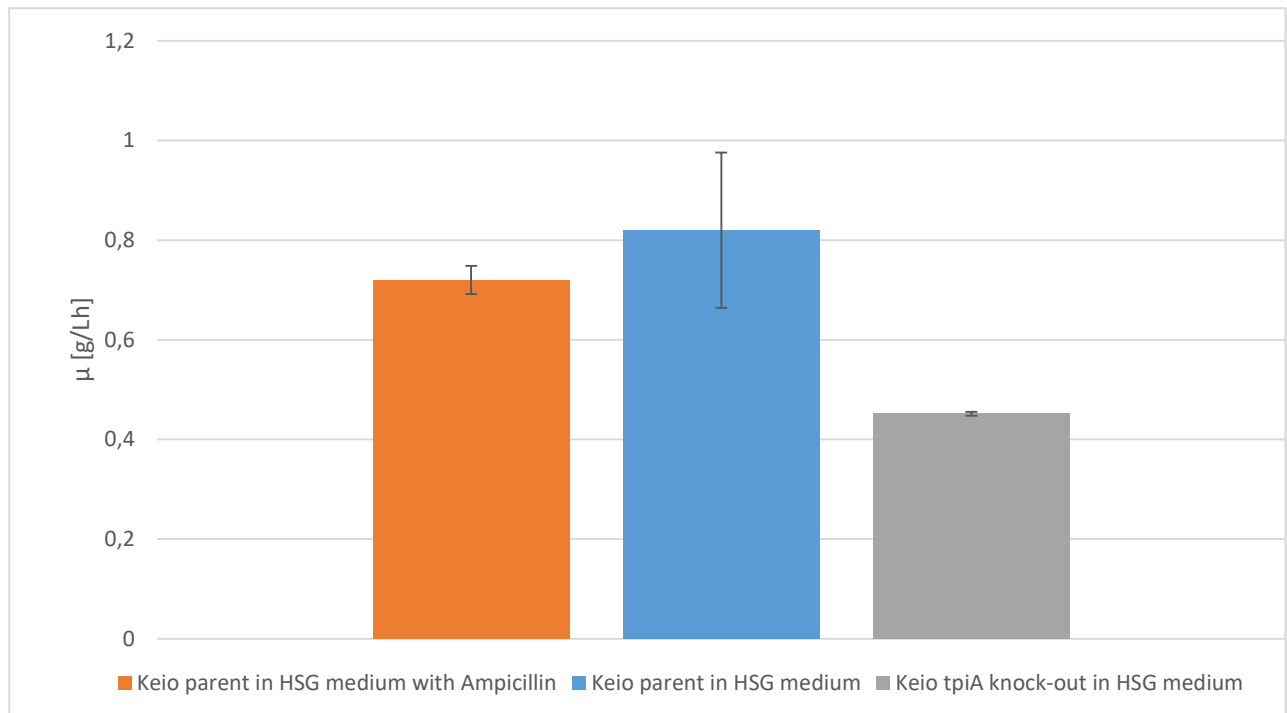


Figure 10. Specific growth rate of cells during cultivation in shake flasks

As it is expected cells grow faster in medium without antibiotic and culture reaches higher cell density, but the problem is that in that medium it is less likely that only desired strain that is growing and it is more likely that there are some contaminations or that plasmid is no longer present in the culture.

Keio tpiA knock-out transformed with pGFP-tpiA, shows lower growth rate in comparison to the transformed parent strain. This cultivation should be repeated for longer time to be sure when the strain reaches stationary phase.

4.1.2. Fluorescence

In order to check what is happening with the plasmid in the culture plasmid stability was measured. As plasmid pGFP-tpiA contains gene for sfGFP plasmid stability was measured through fluorescence. Figure 11. shows how fluorescence changes during cultivation in shake flasks.

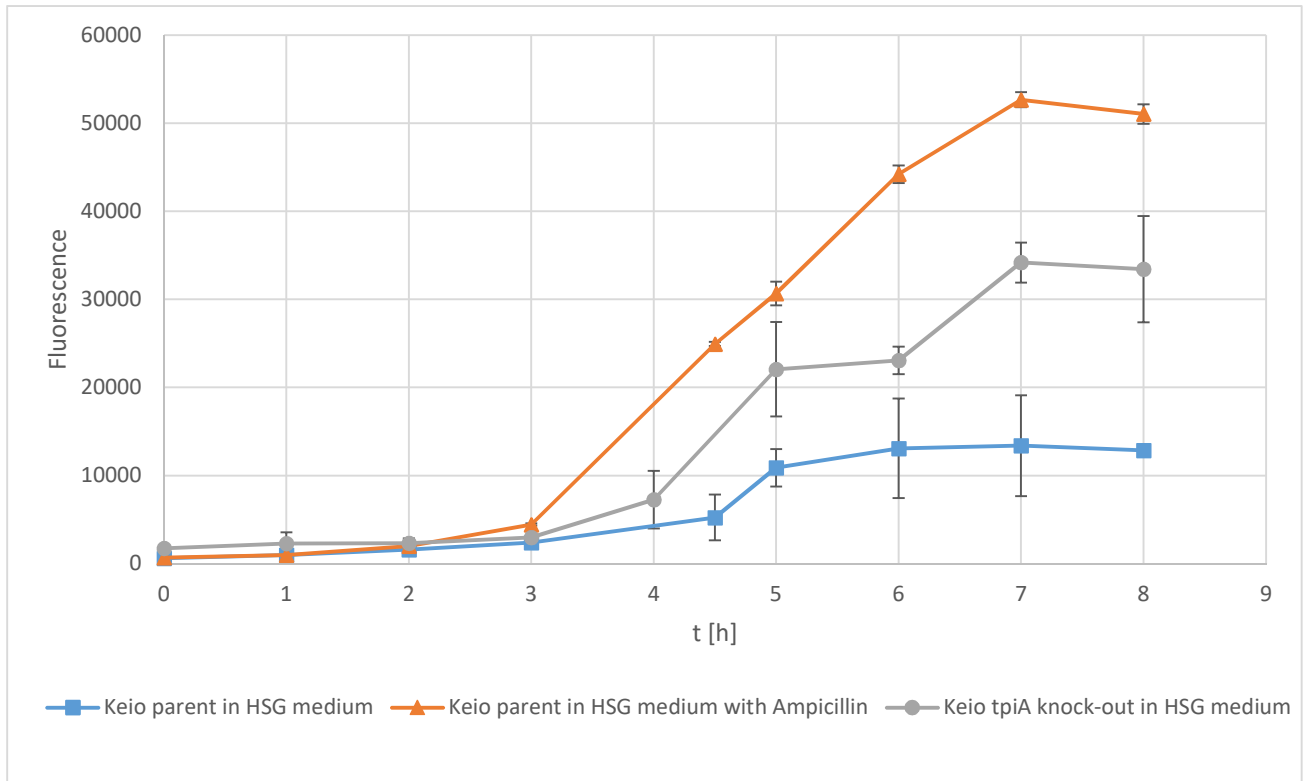


Figure 11. Changes in fluorescence during cultivation in shake flasks

In the beginning fluorescence is low in all variants of fermentation but as soon as the cells get into exponential growth (in the third hour), difference can be seen and it is getting bigger as the cultivation is going further. Plasmid pGFP-tpiA contains *bla* gene for β -lactamase which gives it resistance to Ampicillin and as it is expected that plasmid stability is higher in cultivations with Ampicillin for transformed Keio parent strain. In medium without antibiotic it is not necessary for cells to carry the plasmid and as soon as cells with very low plasmid copy numbers show up, preferentially during high grow rates, when cells might have not enough time to synthesize plasmids in high copy numbers, they will have advantage. Caused by the additional metabolic burden, plasmids may be lost completely, since plasmid-free cells would gain a growth advantage in comparison to plasmid-bearing cells (Velur Selvamani et al., 2014).

Carbon source in HSG medium is glycerin, and bacteria needs enzyme tri phosphate isomerase (tpiA) to grow on it. Knock-out strain, without this enzyme, can grow on this medium only when it has plasmid carrying the correct gene for tpiA. As it is expected, this selection pressure keeps the plasmid stability high during cultivations. (Velur Selvamani et al., 2014).

The difference in fluorescence reached after 8 hours of cultivation in shake flasks can be seen on Figure 12. Fluorescence reached with transformed Keio tpiA knock-out is three times higher compared to fluorescence reached with transformed parent strain cultivated in pure medium, while fluorescence reached in medium with Ampicillin is 4 times higher.

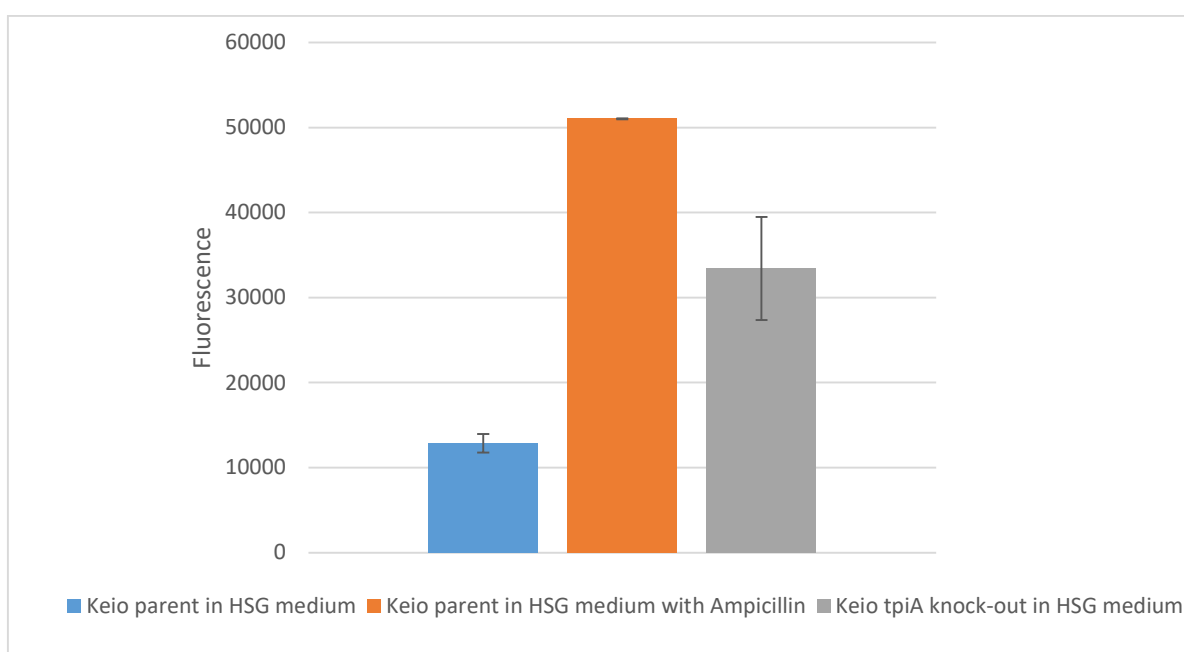


Figure 12. Fluorescence after 8 hours cultivation in shake flasks

4.2. Batch cultivation

4.2.1. Cell growth

Figure 13. shows growth curve for cells cultivated in batch fermentation in bioreactor NLF3.

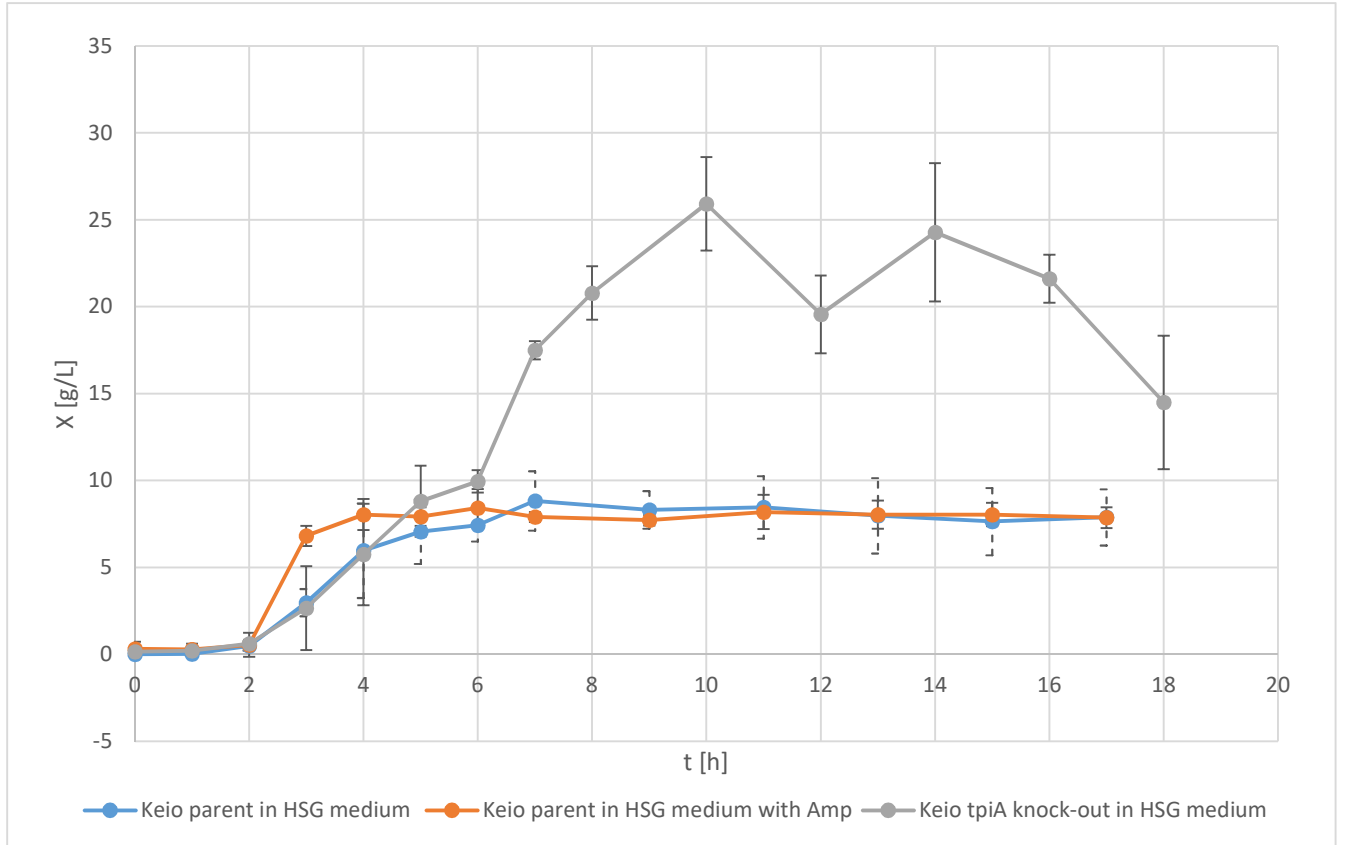


Figure 13. Growth curve of Keio parent and Keio knock-out strain, both transformed with pGFP-tpiA, during batch cultivation in bioreactor NLF3

In this case of transformed Keio parent strain, cells grow faster in the exponential phase faster in medium with Ampicillin, otherwise stated, their specific growth rate is higher during exponential phase, as it can be seen in Figure 14. Transformed Keio tpiA knock out strain in the beginning grows slower than the transformed parent strain cultivated in medium with antibiotic but goes through the phases of growth simultaneously as the transformed parent strain. Transformed parent strain goes to stationary phase in 6th hour, while transformed knock-out strain continues to grow exponentially for four more hours. Cell density reached with the transformed parent strain is around 8 g/L while one reached with the transformed knock-out strain is 25 g/L.

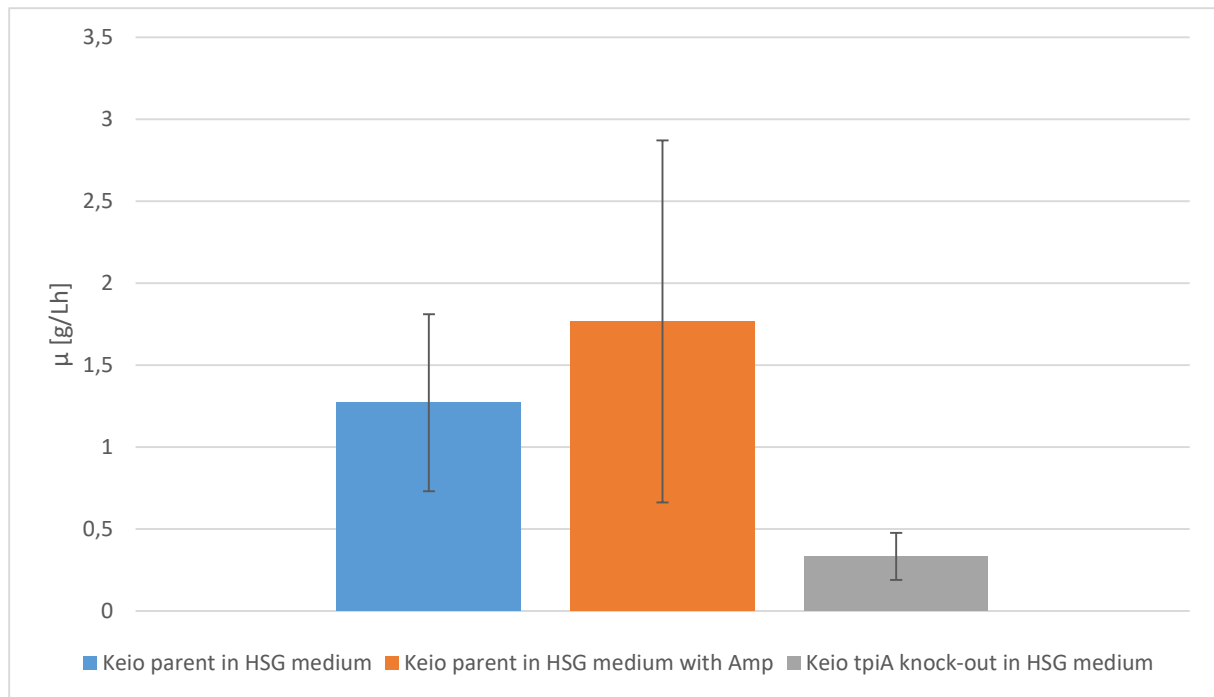


Figure 14. Specific growth rate of cells during batch cultivation in bioreactor NLF3

4.2.2. Fluorescence

Fluorescence measurements during batch cultivation with transformed parent strain are shown in Figure 15. In this case there is no big difference in fluorescence between culture grown in plain medium and culture grown in medium with Ampicillin. Possible reason for it is that preculture was grown in medium with antibiotic in which cells already produced β -lactamase, which then, when added to the bioreactor, degraded the antibiotic present and then there was no need for the cells to keep the plasmid. Ampicillin can be degraded by β -lactamases in less than 30 minutes in high cell density cultures (Jung et al., 1988). Also appearance of plasmid-free segregants is not totally prevented, since the concentration of the antibiotic used for the maintaining of plasmid stability often decreases during long term cultivation as a result of dilution and/or enzymatic degradation by the growing cells (Hägg et al., 2004). On the other side, fluorescence reached with transformed knock-out strain (Figure 16) is more than 200 times higher than one reached with the transformed parent strain, as it can be better seen on Figure 17. So it can be concluded that knock-out strain provides good plasmid stability as an alternative to antibiotic makers.

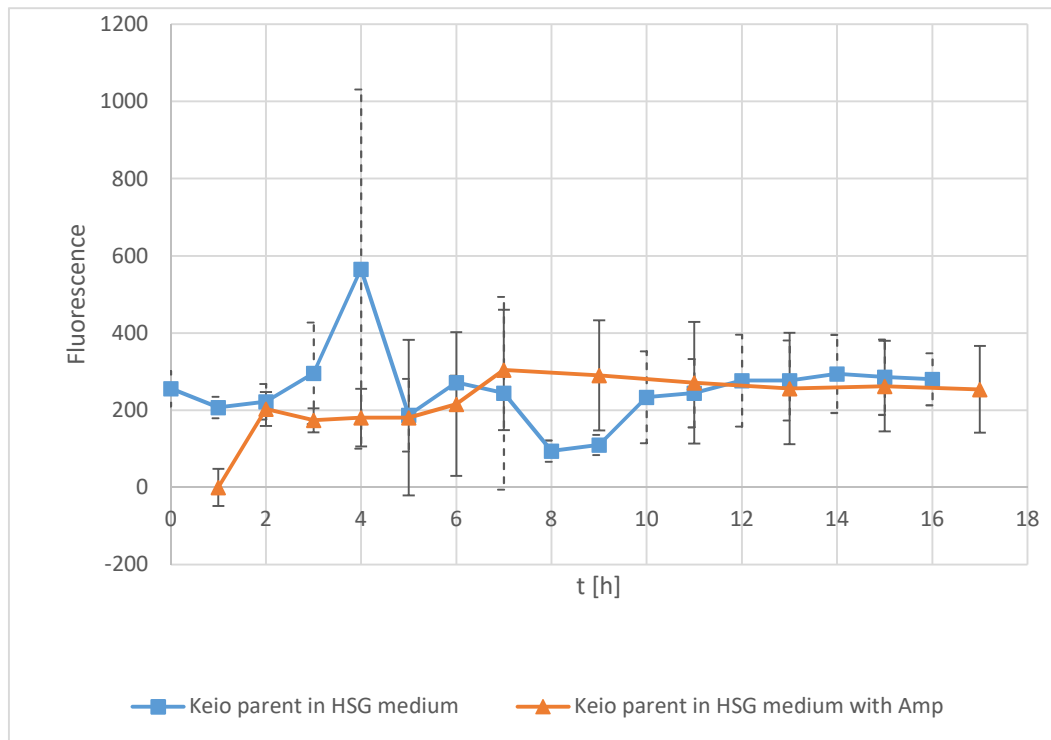


Figure 15. Change of fluorescence during batch cultivation in bioreactor NLF3

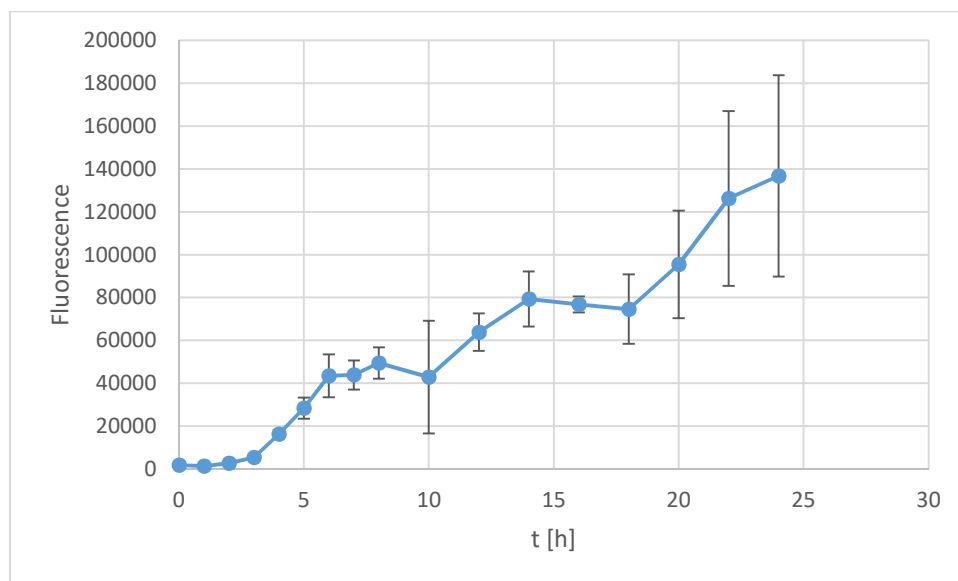


Figure 16. Change of fluorescence during batch cultivation of Keio *tpiA* knock-out strain during batch cultivation in HSG medium in bioreactor NLF3

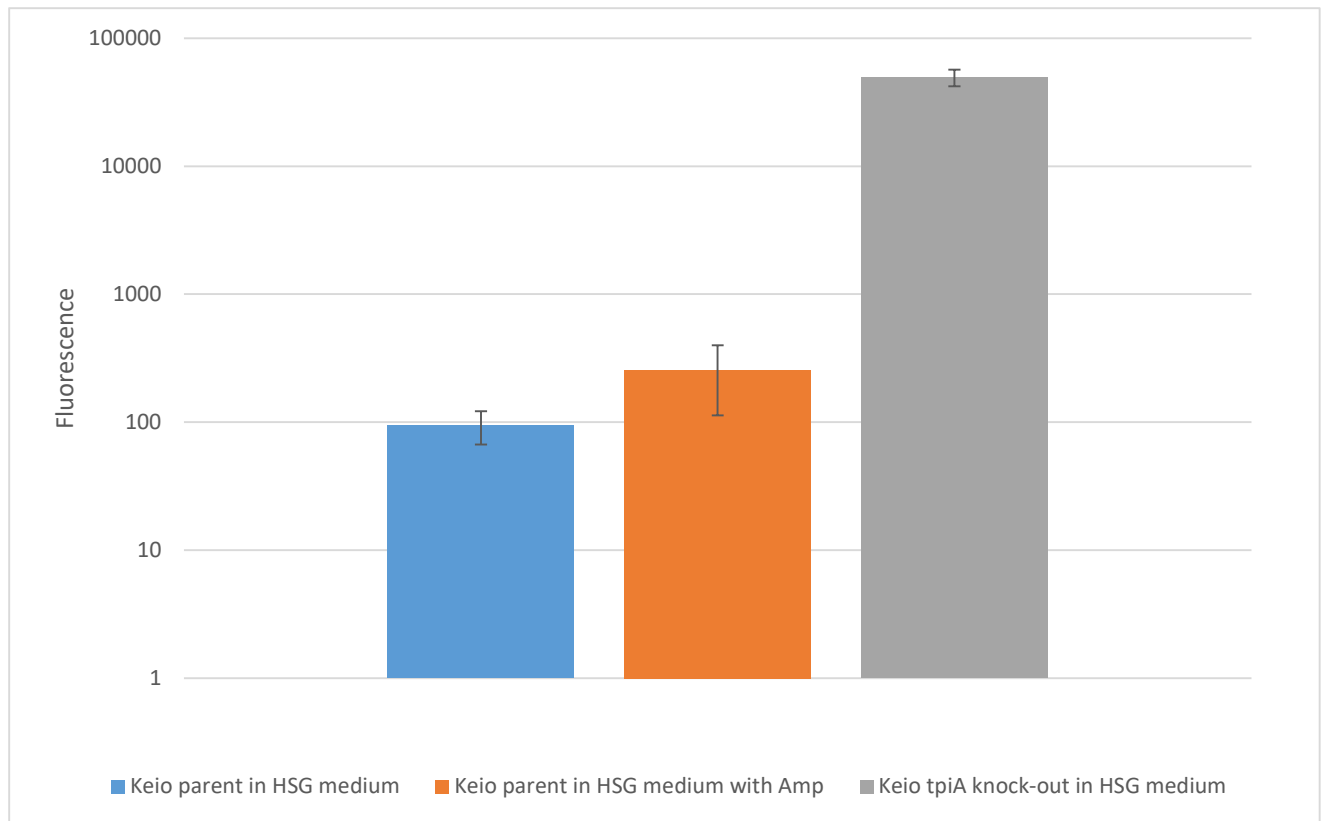


Figure 17. Fluorescence reached after 8 hours of batch cultivation showed with a logarithmic scale

Finally, if we compare fluorescence reached in shake flasks and in bioreactor cultivation with transformed parent strain, fluorescence in shake flasks is 20 times higher in medium without antibiotic and even 100 times higher in medium with antibiotic. On the other hand, fluorescence reached with the transformed knock-out strain is 5 times higher in batch cultivation compared to shake flasks.

4.3. Fed-batch cultivation

After good results obtained with cultivation of Keio knock-out strain, transformed with pGFP-tpiA in batch fermentation, it was repeated with fed-batch to see how growth and plasmid stability changes in high density cultures over longer period of time. Figure 18 shows its growth curve.

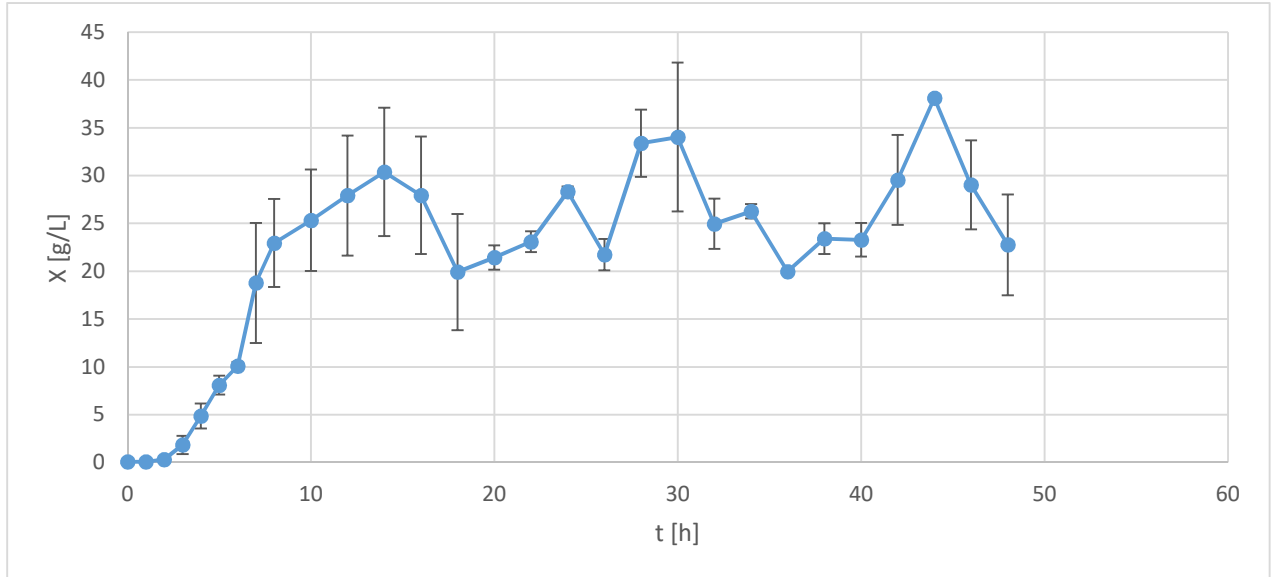


Figure 18. Growth curve of Keio tpiA knock-out strain, transformed with pGFP-tpiA, during fed-batch cultivation in HSG medium in bioreactor NLF3

Cell density reached during fed-batch cultivation is around 40 g/L. If we compare behavior during growth it is similar to batch only that cell grow over longer period because of the addition of fresh media. Specific growth rate during exponential phase is $0,8 \pm 0,13 \text{ h}^{-1}$ which is considerably higher than in batch cultivation ($0,33 \pm 0,14 \text{ h}^{-1}$).

Change of fluorescence during cultivation can be seen on Figure 19. Fluorescence reached during fed-batch cultivation is similar to those reached during batch cultivation. From the graph it can be concluded that plasmid stability is high during cultivation with a slight decline in the end of cultivation. Possible problem is that this knock-out strain still contains some parts of the tpiA gene, its promoter and terminator. This brings up the possibility of recombination between plasmid and cells genome, which could create revertants that would grow on HSG medium without plasmid, and probably outgrow plasmid-carrying cells. But to make further conclusion this cultivation should be repeated for longer period of time and as a continuous culture.

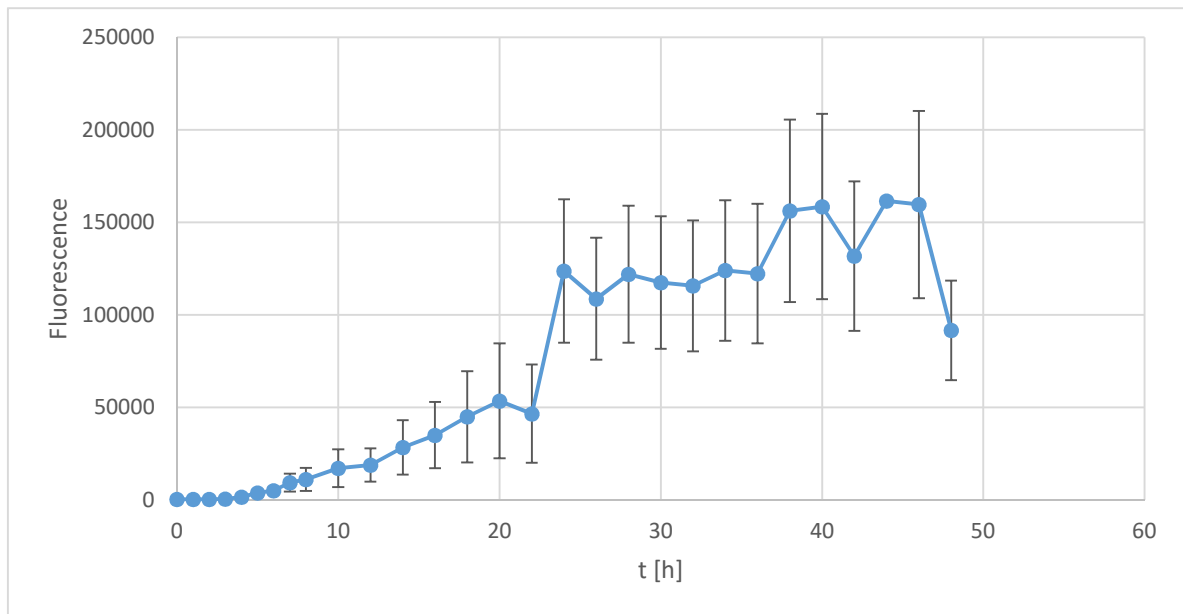


Figure 19. Change of fluorescence during fed-batch cultivation of Keio *tpiA* knock-out strain transformed with pGFP-*tpiA*, during fed-batch cultivation in HSG medium inbioreactor NLF3

4.4. Plating

During cultivations with both Keio parent and knock-out strain, transformed with pGFP-tpiA, sterile samples were collected and plated on LB-Amp agar plates to see how their appearance and fluorescence changes during time. Plates photographed under UV light are shown on Figure 20.

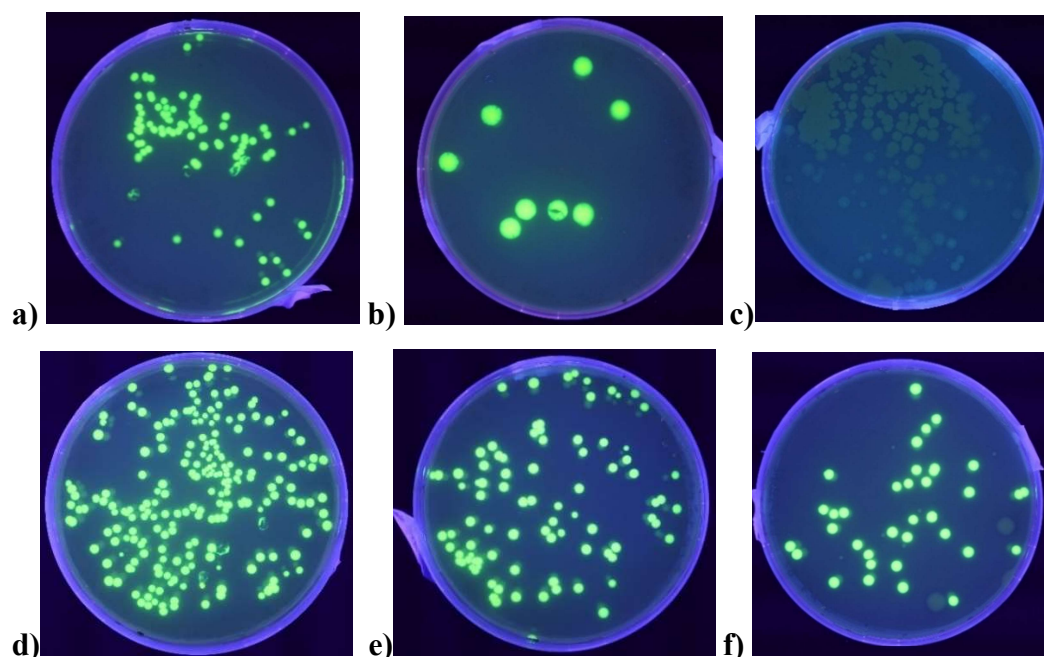


Figure 20. LB-Amp agar plates under UV light after cultivation of Keio parent strain with pBR322-P100sfGFP-tpiAO for 1 (a), 24 (b) and 48 (c) hours and cultivation of Keio tpiA knock-out strain with pBR322-P100sfGFP-tpiAO after 1 (d), 24 (e) and 48 (f) hours

As we see from the pictures transformed Keio parent strain loses fluorescence after 48 hours but it is still resistant to Ampicillin. The question here is if it became resistant to the antibiotic by homologous recombination and lost the plasmid with the gene for sfGFP or the sample was contaminated with the Ampicillin resistant strain. Keio tpi-A knock-out keeps fluorescence even after 48 hours of cultivation.

4.5. Creating a new Keio knock-out strain

The Keio collection is comprised of 3985 strains with deletions in duplicate of *E. coli* K-12 strain BW25113. The problem with the deletions is that all Keio deletion mutants contain 21 bp of the 3' end of the deleted gene, terminator and promoter region and ATG codone. This could easily lead to a re-integration of the deleted gene, when present completely on a plasmid like it is with the *tpiA* on pGFP-*tpiA*.

To lower the possibility of a homologous recombination between the *tpiA* gene on the plasmid and parts of the *tpiA* gene, its promoter and terminator, present in the Keio *tpiA* knock-out strain, new knock-out strain was created from Keio parent strain. In this case, new knock-out strain doesn't contain any of the parts of the *tpiA* gene (Figure 21).

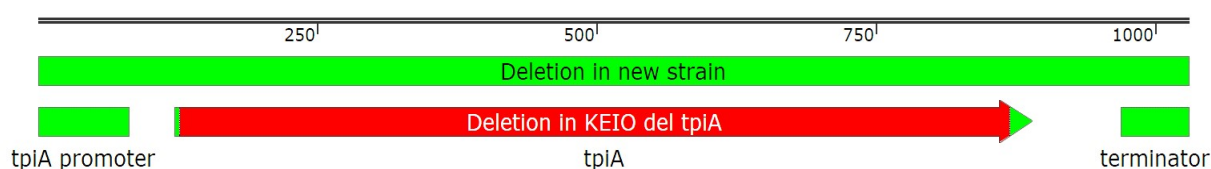


Figure 21. Comparison of *tpiA* deletion between Keio *tpiA* knock-out and new *tpiA* knock-out

Deletion of chromosomal *tpiA* locus was done using the one-step chromosomal gene inactivation technique described by Datsenko and Wanner, 2000. The disrupted gene for *tpiA* was replaced with gene for Kanamycin resistance, with the length of 805 base pairs. Deletion has been proven through gel electrophoresis after PCR amplification of fragment between bases upstream and downstream of previous *tpiA* gene. Gel photographed under UV light is shown on Figure 22.

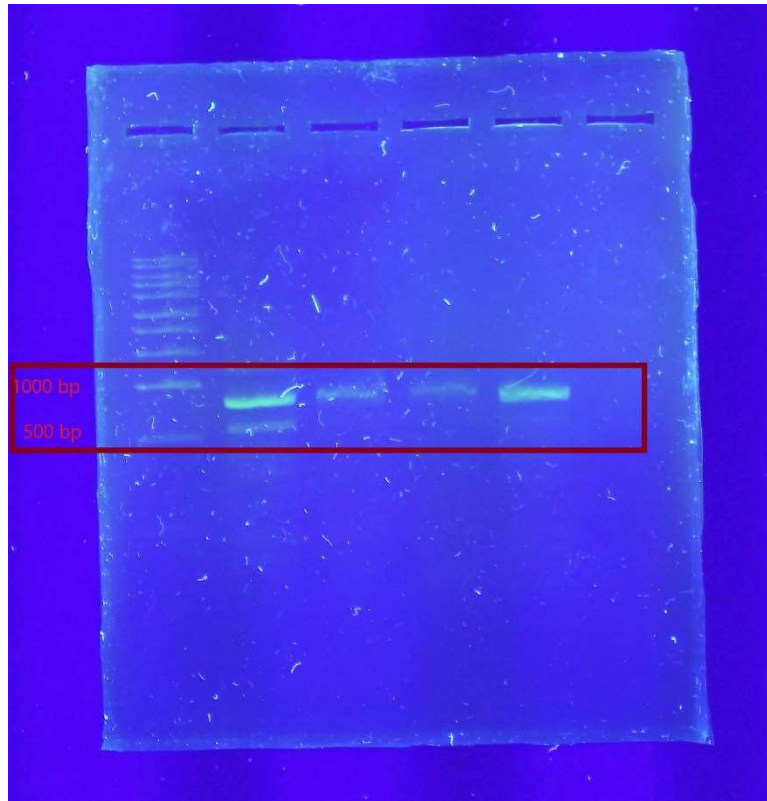


Figure 22. Gel electrophoresis of Kan^R fragment in new Keio *tpiA* knock-out photographed under UV-light

5. CONCLUSION AND OUTLOOK

Within scope of this thesis, growth of two strains, *Escherichia coli* BW25113 and *Escherichia coli* JW3890-2, both transformed with pBR322-P100sfGFP-tpiAO, was observed as well as the plasmid stability. Transformed *Escherichia coli* BW25113, also known as Keio parent strain, was cultivated in HSG medium with and without antibiotic, in shake flasks and as batch fermentation in bioreactor. Transformed *Escherichia coli* JW3890-2, Keio tpiA knock-out strain, was cultivated in HSG medium, in shake flasks, as batch and as a fed-batch fermentation in bioreactor.

1. In shake flasks, growth of the Keio parent strain, transformed with pGFP-tpiA is similar in both variants of fermentation while in medium with antibiotic it is a bit slower. Keio tpiA knock-out, transformed with pGFP-tpiA, needs more time to get to the exponential phase and grows with a lower growth rate but after 8 hours reaches similar cell density as the parent strain.

Its fermentation should be repeated for longer time to observe when it would reach stationary phase and how high would the cell density be in the end.

2. On the other side plasmid stability during shake flasks cultivation, observed as fluorescence, is highest with transformed Keio parent strain cultivated in HSG medium with antibiotic. With transformed Keio tpiA knock-out fluorescence reached is 65 % of fluorescence reached with Keio parent strain cultivated with antibiotic, but it has advantage as antibiotic is not needed for it. Fluorescence reached with transformed parent strain in pure medium is only 25 % of fluorescence reached in medium with antibiotic respectively 38 % of fluorescence reached with transformed mutated strain.
3. During batch cultivations, transformed Keio tpiA knock-out strain has the lowest growth rate but it grows for a much longer time and reaches high cell densities (25 g/L), three times more than the transformed parent strain. Growth of transformed parent strain is similar in both variants of the fermentation, with and without antibiotic.
4. Plasmid stability of the transformed parent strain during batch cultivations is really low, and fluorescence reached is more than 100 times lower then fluorescence reached with the transformed knock-out strain. Plasmid stability of the knock-out

strain, transformed with pGFP-tpiA, is really high and gives good alternative to the use of antibiotics in the medium.

5. In fed-batch cultivation, transformed knock-out strain grows with a higher growth rate and reaches higher cell densities but fluorescence reached is similar to those reached with batch cultivation.

For further research growth and plasmid stability in continuous culture should be observed.

Finally, new strain was created, from Keio parent strain, with deletion of the whole tpiA gene. For further research it should be transformed with pBR322-P100sfGFP-tpiAO, and cultivated in various volumes, from shake flasks to bioreactor, as a batch, fed-batch and continuous culture to observe its growth and plasmid stability and compare it with results obtained from cultivation in this thesis.

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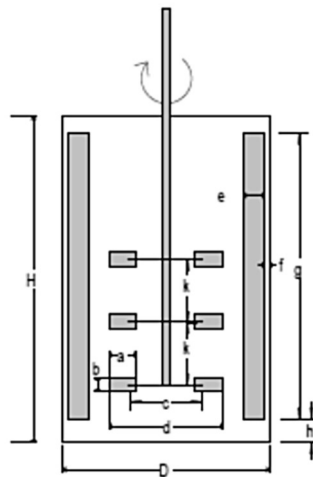
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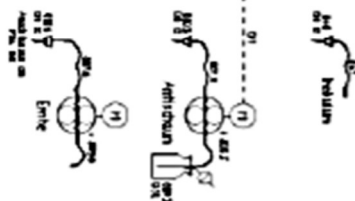
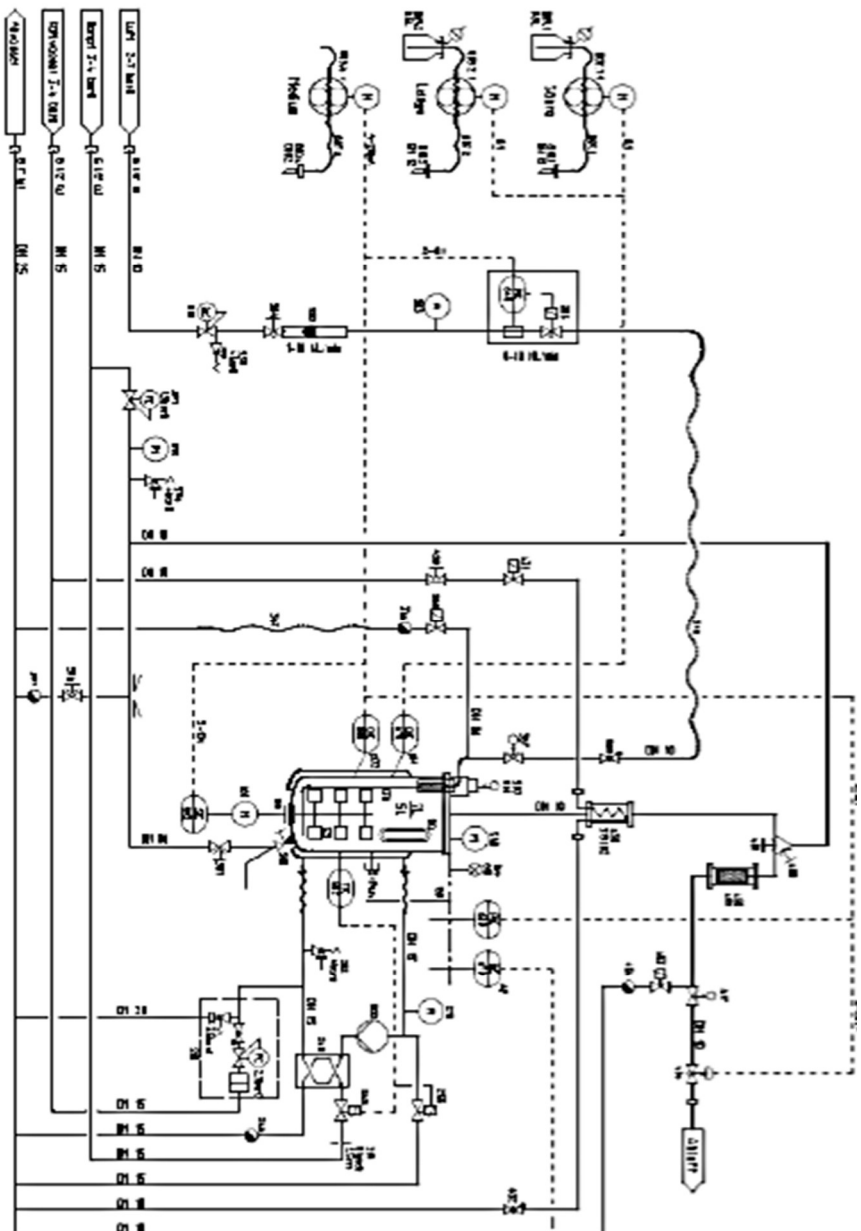
7. APPENDIX

7.1. Bioreactor specification

Bioreactor model	Bioengineering NLF 1
Process control	BioSCADA Bioengineering
Total volume	7 L
Reactor diameter (D)	155 mm
Reactor height (H)	360 mm
Impeller diameter (d)	60 mm
Impeller blade width (a)	21 mm
Impeller blade height (b)	16 mm
Blade diameter (c)	30 mm
Number of blades	6
Number of stirrer levels	3
Blade bottom and bioreactor bottom distance	70 mm
Distance between two stirrers (k)	122 mm
Distance between the second stirrer and bioreactor bottom	65 mm
Stirrer blade and air opening	60 mm
Stirring shafts diameter	12 mm
Number of baffles	4
Baffle height (g)	326 mm
Baffle width (e)	15 mm
Baffle and bioreactor wall distance (f)	3 mm
Baffle and bioreactor bottom distance (h)	0 mm



7.2. NLF 3 bioreactor system scheme



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IZJAVA O IZVORNOSTI

Izjavljujem da je ovaj diplomski rad izvorni rezultat mog rada te da se u njegovoj izradi nisam koristio/la drugim izvorima, osim onih koji su u njemu navedeni.

Lukrecija Mahalic

Ime i prezime studenta